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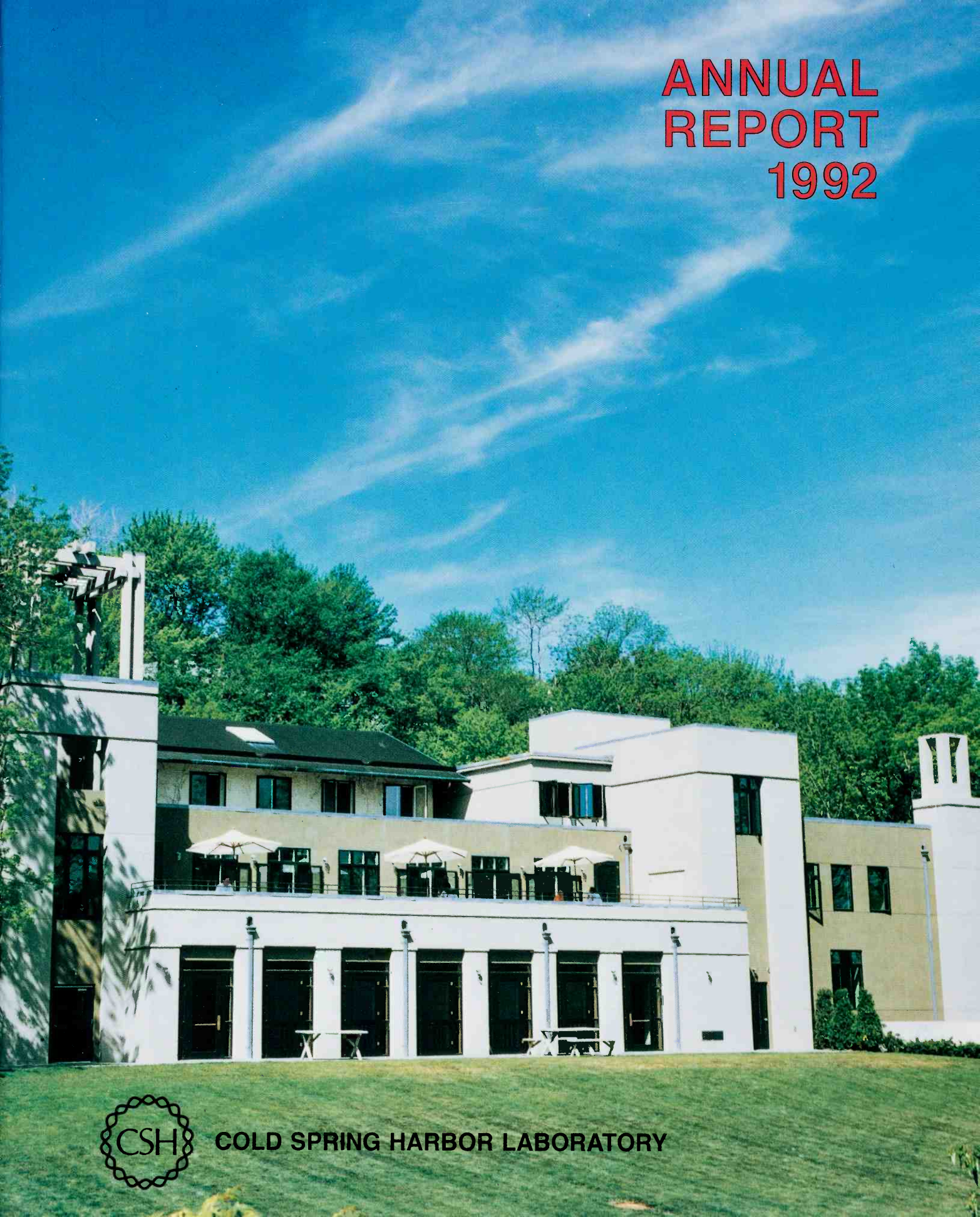

COLD SPRING HARBOR LABORATORY

ANNUAL REPORT 1992

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Back cover: Aerial view of Cold Spring Harbor Laboratory (Photograph by Margot Bennett)

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COLD SPRING HARBOR LABORATORY

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Barbara McClintock

1902–1992

"Private Residence. Do Not Enter." These words, written in a plain hand on a small, aged, brown card thumbtacked to the door of Barbara McClintock's Hooper apartment, must have dissuaded many would-be proselytes. Others were intimidated by her presence. Although nearly everyone at the Laboratory in the last 50 years saw Barbara somewhere, in her laboratory, strolling the grounds, or at Laboratory functions, not everyone spoke to her. Striking up a conversation with the legendary scientist required either courage or nerve. Small in size, of the minority gender in science, and averse to publicity, Barbara protected herself from outsiders with a reputation of reclusiveness. But those who raised a feather to this wall found it easily toppled. Behind it was a woman of great warmth, boundless intellectual energy, and open mind. She was willing to talk to anyone who really had something to say; her only intolerances were of stupidity and zealotry.

Barbara was born on the 16th of June, 1902, in Hartford, Connecticut. Her parents, Sara Handy McClintock and Thomas Henry McClintock, were both from New England, but they had very different backgrounds. Barbara's mother came from one of the best familes in Hyannis, Massachusetts, and could trace her lineage on both sides back to the Mayflower. Her father was the son of British emigrants, a newcomer by his future in-laws' standards. When they were married, Thomas was still in medical school, and so the young couple had little money. They soon began having children, however, and Barbara was the third of four children.

When Barbara was six, the family moved to Brooklyn. Barbara attended Erasmus Hall High School, and at age 17, she entered Cornell University's School of Agriculture in Ithaca, New York. She majored in botany, receiving her B.A. degree in 1923. Advanced degrees followed in quick succession: She earned an M.A. in 1925 and a Ph.D. in 1927. In the mid 1920s, genes were still abstract units of heredity. Geneticists widely accepted that genes were carried on the chromosomes, but the bricks and mortar of a gene, its physical basis, was not known. Barbara's dissertation was on cytogenetics, a new field that sought to link patterns of inheritance to the behavior and structure of chromosomes. Her organism of choice was *Zea mays*, or maize, the colorful "Indian corn" familiar in Thanksgiving table settings. Maize was one of two primary organisms used in genetics at that time, the other being the fruit fly *Drosophila*, whose use was pioneered by Thomas Hunt Morgan. Maize was useful because of the pigments that make its ears so appealing. The color patterns these pigments produce can be easily tracked, both through the development of a particular ear and from generation to generation. The problem with maize, however, was that identification of the individual chromosomes had so far been impossible. Barbara's thesis described a technique for staining and distinguishing each of the ten maize chromosomes, thus making possible studies linking the inheritance of specific traits to a given chromosome. Barbara's studies in maize, carried out with Harriet Creighton, were among the first in biology to show that genes are carried on chromosomes. This paralleled the famous *Drosophila* work in Thomas Hunt Morgan's laboratory that resulted in the "chromosomal theory of inheritance."

Barbara's Ph.D. adviser was R.A. Emerson, one of the leading cytogeneticists of the day. Her colleagues at that time included Marcus Rhoades, who became one of the foremost maize geneticists of the last 50 years, and George Beadle. Beadle later left maize genetics to study *Drosophila* with Morgan and then switched to the mold *Neurospora*. In 1958, Beadle won the Nobel prize for his role in developing the "one gene–one enzyme hypothesis" that helped usher in the new field of molecular biology. Emerson thus has the unusual distinction of having trained two future Nobelists simultaneously.

Barbara remained at Cornell for several years after completing her Ph.D., working as Emerson's research assistant and pursuing her studies in maize. Always a bright student, it was during this time that she began her long series of uncanny insights into the behavior of genes and chromosomes. From the behavior of certain traits of pigmentation, Barbara inferred the existence of small ring chromosomes. Throughout the cell division cycle, these rings would break and replicate, randomly losing certain pigment genes in the process. She

became convinced of the existence of ring chromosomes based on her reasoning, and only then did she actually see them in her histological preparations. It was this ability to think through her data, rule out all possibilities but one, and "know" how the chromosomes must be acting—even when they were acting in ways contrary to everything then known about them—that distinguished Barbara's science.

In 1936, Barbara was appointed assistant professor at the University of Missouri, Columbia. There, she generalized her work on broken chromosomes and described the "breakage-fusion-bridge" cycle. Barbara noticed that under certain conditions, her chromosomes would break, forming loose ends that could fuse with like or unlike chromosomes. Two of these mix-and-match chromosomes sometimes formed a bridge, which stimulated another breakage and refusion, beginning the cycle again. By repeatedly cutting and pasting pieces of chromosomes to each other, the breakage-fusion-bridge cycle shuffled the pigment genes, producing new patterns in the maize kernels. This was the first of several major contributions Barbara made to understanding how the genome introduces variety within itself. In genetics, variety is not the spice of life, it is the stuff of evolution.

The prejudices of the day created not a "glass ceiling" for women scientists, but a concrete one. Barbara was told outright that her chances of being promoted at Missouri were minimal, even though she had become a well-respected scientist and had served as the vice president of the Genetics Society of America. At the end of 1941, she accepted an invitation from the Carnegie Institution of Washington to join its Department of Genetics at Cold Spring Harbor. At the turn of the year, she was made a staff member of the Carnegie, a position she held until 1967, when she became a Distinguished Service Member.

It was at Cold Spring Harbor that Barbara made her most famous contribution to science. Using the same skills she had used in identifying ring chromosomes 20 years before, Barbara reasoned the existence of a brand-new phenomenon in chromosome behavior. To set the scene, the prevailing view of the genome at that time was a static one. A chromosome was a string of genes, each fixed in position relative to every other. When Barbara announced, in 1951, that she had found genes that seemed to move around, it was as surprising as if the big pearl in the center of a necklace spontaneously jumped to a position near the clasp. These movable genes, called transposable elements or transposons by scientists and "jumping genes" by the popular press, shook the foundation of the prevailing view of the genome.

This story has been told so many times that it has become a myth. Barbara has been canonized as a martyr of scientific dogmatism and narrow-mindedness. Her image in the public eye is that of a diminutive underdog vainly trying to speak the truth to the entrenched old-boy network of scientists, a woman ignored for 30 years until more objective and enlightened young minds gave her the credit she deserved. In fact, Barbara was already a giant in the field when she discovered transposons. She had been a member of the National Academy of Sciences for seven years. She had published many important papers. Although it is true that the significance of her work was not widely appreciated for a long time, the reasons are more subtle.

First of all, Barbara's work was hard to understand. She thought in such leaps that even many first-rate minds had trouble following all of her arguments. The brilliant *Drosophila* geneticist Alfred Sturtevant reportedly said of this work, "I don't understand a word of it, but if Barbara says it is so, it must be so!" Barbara, after all, had never actually seen a transposon. She had again inferred their existence from careful study of her crosses. Few could follow the intricate lines of reasoning that led her from splotches of color on corn kernels to bits of genes hopping around the chromosomes. Second, its applicability to other organisms was not clear. Many people thought that Barbara was probably right, but so what? It was likely a genetic curiosity, restricted to maize. Plants were, after all, rather strange organisms. Few people expected to find transposons in bacteria or yeast, let alone humans.

It took the advent of molecular biology to make clear the existence and importance of transposable elements. In the late 1970s, science had advanced far enough that we could

isolate the actual DNA that jumped from site to site in the genome. Nina Fedoroff studied the molecular biology of maize transposons, at last isolating the genes Barbara could see only in her mind. Also, transposons were found in other organisms. David Botstein found transposons in bacteria. Gerald Fink found them in yeast. It was the generality of transposition that won Barbara McClintock the Nobel prize, and it took time for that generality to be established.

Following her transposon work, Barbara took maize research into yet another new arena. She undertook a sort of agricultural anthropology, using maize genetics to trace the ancient, sophisticated Indian cultures of Mexico. For this work, she traveled to Mexico, learned Spanish, and discovered a new meaning for the term "field work."

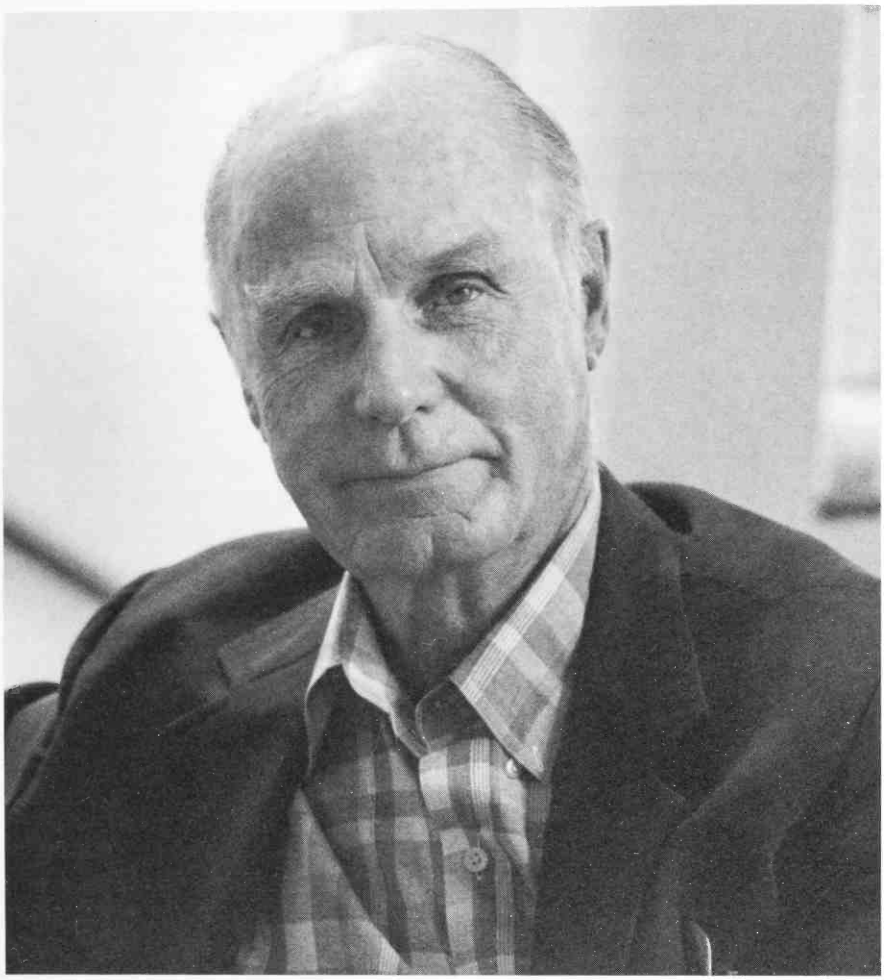
Barbara lived and worked at Cold Spring Harbor for more than 50 years—nearly half the Laboratory's history. During that time, she had a significant influence on Cold Spring Harbor science. She may have influenced younger generations of scientists even more than her contemporaries. She exerted a direct influence, of course, on the plant geneticists here. Some are exploring the molecular biology of Barbara's famous *Ac/Ds* system. Others are using transposons as vehicles for inserting "reporter" genes into an otherwise unwilling host, the chromosomes of the small plant *Arabidopsis.* When a transposon ferries a reporter into an *Arabidopsis* gene, scientists can identify both the location and the function of the *Arabidopsis* gene. Barbara spoke with Laboratory scientists often, giving her advice, visiting the cornfields at Uplands Farm. Her work of the 1950s and 1960s was important to the yeast group here during the 1970s, as they worked on mating-type switching. Barbara's early work on the breakage-fusion-bridge cycle identified the structures at the chromosome tips now called telomeres, sparking a field of research that has implications for both aging and cancer.

A striking feature of Barbara's research is that it continues to generate new ideas. In molecular biology, knowledge advances so quickly that a paper becomes "old" (i.e., irrelevant) in 5 years or less. Yet Barbara's papers from the 1960s, 1950s, 1940s, even the 1930s continue to be cited. The reason is that she sought "molecular" explanations for her observations even though the actual molecules had not yet been identified. She saw a level of abstraction deeper than her data could take her. As the science catches up with her thinking, the relevance of her interpretations becomes clear.

Barbara's main influence on Cold Spring Harbor, however, was on a one-to-one level. People from all sectors of the Laboratory—Rolling Five scientists, postdocs, administrators, and groundskeepers—had ongoing conversations with her. They talked about science, wildflowers, politics, the weather, where to find a good sweater. With the younger scientists, she often talked about motivation and the life of a researcher. Barbara had a reputation for being a mystic. She wasn't a mystic, she was just thoughtful. She was interested in what a person had to say. She would ask questions until she understood the problem and then draw on her experience to give a fresh perspective. If she saw things the rest of us did not, it was simply because she thought more clearly and deeply than most of us.

Since Barbara's death on September 2, 1992, the Laboratory has not been the same. For many of our community neighbors, Barbara McClintock symbolized Cold Spring Harbor Laboratory. People with no idea of what an oncogene or a receptor protein is knew about "the corn lady" who somehow beat the system and eventually got the recognition she deserved. She has become a local legend. Ironically, Barbara would just as soon not have been a legend, not a Nobel laureate, not famous. It was fame that forced her to hang that sign on her apartment door. Those who cared about who she was, rather than what she did, knew that below the words "Do not enter" was an implicit "Please knock."

Nathaniel C. Comfort



Taggart Whipple

1912–1992

The Laboratory has three overlapping types of supporters: those who contribute scientifically, those who contribute monetarily, and those who contribute with their spirit. Had Taggart Whipple been a scientist, he would have been all three. As it was, Tag, who passed away on November 26, 1992, was a long-time friend and adviser who left an indelible mark on Cold Spring Harbor Laboratory.

Born in Manchester, New Hampshire, in 1912, to Stanley Cornell Whipple and Ruth Storey Whipple (née Taggart), Tag was raised in the Massachusetts town of Cohasset, near Boston. His father sold advertising for major magazines such as *The Saturday Evening Post.* He attended the small, private Noble and Greenough School, where he did well and graduated young. His parents thought it would be best if he attended a somewhat larger prep school before he went on to college, so he attended Exeter for a year after graduating from Noble and Greenough, an experience he thoroughly enjoyed.

In 1929, Taggart matriculated at Harvard. He did well academically and also took part in sports and social activities. After injuring his knee playing football, he became a member of the rowing team. He graduated in 1934 with a bachelor's degree in English.

After graduation, Arthur Chase, a rowing buddy, invited Tag to spend time with him at his Adirondack mountain camp. As it happened, a young woman from New York City named Katherine Brewster was summering with her family at their nearby camp. She was learning to sail, to the chagrin of her father, who worried about her out on the water in a tippy boat. When the crewman she had working the mainsheet of her boat had to leave, she began scouring the nearby camps for a suitable crew member. She wandered into the camp of a family friend, Arthur Chase, who said he had staying with him a young man who was about six-foot-two, 170 pounds. Katherine thought, "Hmmm, that sounds about right." Taggart was willing, so she had herself a full crew.

At the end of the summer, Katherine and Tag went their separate ways and did not see each other for several years. Taggart went to New York to begin work and school. He had hoped to attend Harvard Law School, but the Depression had sapped his father's resources so badly that he could not afford to send his son to the prestigious and pricey institution. So Taggart set out on his own to put himself through school. His friend Arthur Chase introduced him to Judge Barrett, at the Lehigh Valley Railroad, who put Tag to work as a law clerk. Tag also attended law school at night at New York University, and by 1938, he had four years experience in a law office, as well as a fresh J.D. degree. That year, he passed the New Ycrk State Bar exam.

Judge Barrett was sufficiently impressed with this young man that upon Taggart's graduation, he arranged an interview for Tag with the New York firm of Davis, Polk, and Wardwell. This first-rank firm drew its members from the best law schools, not from night school. Around the halls of Davis, Polk, and Wardwell some people wondered what things were coming to if they were beginning to hire NYU night school graduates. Nevertheless, Taggart got the job. He proved to be an intelligent, thorough, and extremely hard worker, and over the next four years, he rose through the ranks. He developed a reputation for leaving no stone unturned when he was preparing a case. He was meticulous and well-prepared—a formidable opponent in the courtroom.

After several years, Taggart and Katherine met again through mutual friends, and in 1941, they married. In the spring of 1942, Taggart was called up for service. He wanted to join the Navy but was turned down because of his college knee injury. He wound up in the Army, in Ordnance, repairing equipment as it returned from the Pacific. But Taggart wanted to go to the Pacific. After pestering his commanding officer repeatedly about a transfer, the CO finally exploded, "If you're not quiet, I'll have you transferred all right—to the Aleutian Islands!" Taggart was finally transferred into Field Artillery. He and Katherine moved to Fort Sill, Oklahoma, and then to Texas; then at last, Taggart went overseas, to Italy. Upon his return in 1945, he and Katherine moved to Washington, D.C., where Taggart worked in Intelligence. The Whip-

ples' first child, Christine, was born in Washington in 1946.

Like a lot of young men, Taggart was not quite sure what he wanted to do after the war. He thought about going back to Boston, and he considered moving to Philadelphia. He finally decided he didn't like the way law was practiced in those cities, and so he returned to New York and Davis, Polk, and Wardwell. He spent the rest of his career there and in 1950 he made partner. He distinguished himself through his intelligence, skill, and hard work, as well as through his mentoring ability. Younger lawyers who worked with Tag later wrote that he was tough, fair, thorough, and a role model they kept throughout their careers. He argued before the U.S. Supreme Court on two occasions, and he won one of those cases.

Although he remained with the same law firm for the rest of his career, Taggart's life was far from static. He and Katherine, known to all as "Sinka," adopted two boys, Hugh and George (called Steve). Tag was devoted to his children, and although he may have been strict, he loved them dearly. He was always there to help them out of a scrape when necessary. Like all lawyers in the fast-track world of New York corporations, Taggart worked extremely long and hard hours, yet he was always able to separate his work life from his home life. He didn't bring the office home with him; when he was with his family, he was all theirs.

Since his college days, he loved anything to do with boats and the water (this native bent was undoubtedly reinforced by the way he met his wife). Tag took his family on sailing vacations to Nantucket, Maine, and Shelter Island. The Whipples were friends with Laboratory patrons Walter and Jane Page, and once they vacationed together in the Caribbean, sailing from island to island. He also cultivated an appreciation for the arts. His introduction to art came through a feisty oilman named Charlie Wrightson. Wrightson turned barrels of oil into barrels of money and accordingly had a retinue of attorneys. These lawyers were apparently more eager to keep the Wrightson account than to give him sound advice; so Wrightson fired them and approached Taggart Whipple. Never a yes-man, Tag said that if Wrightson wanted to do something that Tag thought was ill-advised, he would tell him so. Wrightson liked this; he hired Tag and also befriended him. Wrightson had an art collection at the Metropolitan Museum of Art and introduced Tag to the arts. Tag later became very interested in the theater. He became associated with the Lincoln Center for the Performing Arts. He also served on the Board of Directors of the Repertory Theater of Lincoln Center and was a member of the Board of the Lincoln Center for many years.

Walter Page, whose family has been associated with the Laboratory for more than 65 years, introduced Tag to the Laboratory in the early 1980s. Tag quickly took to the way we live and joined our Board of Trustees in 1983, just before his 1984 retirement from Davis, Polk, and Wardwell would provide him much time to work in the Laboratory's behalf. From 1986 to 1992, he was vice chairman of our Board, serving also as chairman of the Nominating Committee and member of the Executive and Development Committees. During our Second Century Campaign, he and Sinka took great pleasure in making possible the construction of the Whipple Room in the Beckman Building, later providing us with a collection of paintings and drawings to decorate its walls.

Always he was the consummate friend and adviser. In particular, I valued Tag's love for the English language and the happiness that came from the use of a word or phrase appropriate to the occasion. Upon becoming a member of our Board, he made a point of reading my past Director's Reports and made me feel good by telling me how interesting they still were years removed from the occasion of the writing. In his last months, when he no longer was effectively mobile, I took much pleasure in bringing him the first drafts of a still not yet completed book on how my family and teachers formed the values that have shaped my later life. Even in his last days, Tag could still spot awkward language while at the same time reassuring me that those who knew me less well would also enjoy reading my new book.

Tag's résumé of memberships reads like a laundry list of the Renaissance Man. Besides his Lincoln Center affiliation, he was a member of the Phoenix Theater, the New York University Institute for Fine Arts, Distillers (USA) Inc., the Council on Foreign Relations, and the

Society of St. Johnsland. In addition to the Laboratory, he was a member of the Woods Hole Oceanographic Institution and the Community Hospital of Glen Cove. Tag also served on numerous educational boards, including those of NYU, the NYU Law Center Foundation, Vassar College, East Woods School (which his children attended), Salisbury School, the Harvard Alumni Association, and the British-American Educational Foundation. His professional associations included the American Bar Association, where he served on the council section on antitrust law from 1966 to 1970; the New York State Bar, where he chaired the antitrust law section in 1962 to 1963; and the New York City Bar, for which he chaired the committee on trade regulation and trademarks. He also served on the Federal Bar Council, the American Judicature Society, and others. In addition, he was a member of the Century, Piping Rock, Union, Pilgrims, and Anglers Clubs.

His many accomplishments by themselves, even a complete catalog, could never sum up Taggart Whipple. His greatest contributions to the Laboratory, to his business, and to his family by their essence are not documentable. At their heart was always his awesome integrity. He made us expect more of both ourselves and the organizations we believe in. Most importantly, he knew how to behave when the inappropriate decision might lead to real trouble. On such occasions, I invariably went to Tag.

Without him, I grieve.

James D. Watson

DIRECTOR'S REPORT

Forty years ago, in Cambridge, England, Francis Crick and I proposed the double-helical structure for DNA, the molecule that carries genetic information. Before we found it, we feared that the structure might be uninformative, still leaving us in the dark as to the essence of the gene. But as soon as we saw the possibility that the structure of DNA could be double-helical, we became enormously excited. The double helix, with complementary sequences of the four bases on its two chains, suggested a simple way for the genetic information of our chromosomes to be exactly copied when our cells divide to form new cells. From the moment we saw the A-T (adenine-thymine), G-C (guanine-cytosine) base pairs, we knew that our double-helical structure, if later validated, would change the way we think of life. By providing a logical starting point to understanding heredity in terms of molecules, Francis and I also suspected that we would go down in history as two of the major names of science. But we knew that the science behind our discovery was not that difficult and that it was the elegant simplicity of the double helix that might make us household names to biology students of the future. So we were not surprised that many scientists in Cambridge who did not think we deserved the resulting fame took pleasure in calling the double helix the *WC structure*.

Our discovery was not all luck, however, since independently we had made two decisions that gave us great advantages over other scientists who also hoped to discover the real secret of life. First of all, both Francis and I thought that DNA had to be the most important molecule of life since it was the molecule of the gene. This opinion was then a minority viewpoint among our peers, and the vehemence with which we held to this belief led many around us to regard us as unsound yet arrogant. If we were right, they might be devoting their lives to minor objectives. Our second conviction was that to understand functions, one had to understand molecular structure at the three-dimensional level. To many others, this was reductionism at its worst, taking away from life the grandeur that they thought it deserved.

Fortunately, both of us could test our beliefs in a then tiny research unit for the study of molecular structure supported by the Medical Research Council (MRC) at the Cavendish Laboratory. The Cavendish Professor at that time was Sir Lawrence Bragg, who early in his youth had discovered how to use X-ray crystallographic diffraction techniques to solve the first three-dimensional structures of simple molecules. For this work, Bragg had received the Nobel prize when he was only 25, and now at 61, he was keen to see his technique used to solve the structure of the key macromolecules of life, the proteins and DNA. At his suggestion, the MRC had set up a small unit, headed by Max Perutz, to work out techniques for solving, in particular, the structure of proteins.

When I arrived in the fall of 1951, there were fewer than ten scientists so supported, but what a powerhouse of brains and common sense. We could think for the long term and not worry about justifying our ideas before scientists who had more pedantic objectives and who would be bound to object that we were wast-

ing government money on projects unlikely to come to fruition in the foreseeable future. To many, Sir Lawrence Bragg was taking a dangerous course in not devoting all of the Cavendish Laboratory to the nuclear physics that had made the Cavendish the most famous of all scientific laboratories in the history of science. But in retrospect, we now can take pleasure that his term as Cavendish Professor had just as great an impact on science as the glorious reign of his immediate predecessor Ernest Rutherford. Not only did Francis and I find the double helix, but it was Bragg's patronage that made possible the 1959 elucidation of the three-dimensional structures of proteins by Max Perutz and John Kendrew.

To follow up these discoveries, the MRC unit required more space than could be provided by the Cavendish Laboratory. At first, a now-famous Quonset hut gave the unit needed breathing space for Francis Crick and Sydney Brenner to tackle the Genetic Code. But soon the decision was made to create, in 1962, a major new MRC Laboratory of Molecular Biology (LMB) on the new Addenbrooks Hospital site. Happily, by continuing to have grand objectives and long-term support, this laboratory, although much larger, is still at the forefront of biology. Four Nobel prizes (Fred Sanger, Aaron Klug, Cesar Milstein, and George Köhler) have already recognized the stellar science accomplished over the interval, and we can confidently expect more to be awarded for research now ongoing. Although daily life no longer has the wonderfully unhurried pace of 40 years ago, the constant preoccupation with writing grants to obtain research monies that now so drags down the American scientific scene is not a part of the LMB scene. One large grant from the MRC keeps it on its grand path.

Now the Cold Spring Harbor Laboratory is roughly the same size as the LMB, and judged from the number of literature citations that our scientific papers receive, we have been almost equally influential in the development of molecular biology over the past several years. Ratings published last year showed that Cold Spring Harbor Laboratory and the MRC Laboratory of Molecular Biology are the two most productive molecular biology laboratories in the world, despite the very different ways our respective science is funded. LMB has had the comfort of stable, long-term government funding, whereas Cold Spring Harbor Laboratory has been almost totally dependent on the receipt of short-term research grants, whose future receipt would quickly be threatened if the quality of our science were to decline.

Such *soft money*, however, was not always the way of life at Cold Spring Harbor. From 1904 to 1962, genetics programs here were maintained by funds provided from the large endowment of the Carnegie Institution of Washington, a body for the support of science created in 1902 by Andrew Carnegie with some of the monies that he obtained from selling his steel mills to the U.S. Steel Company. When the Carnegie Institution of Washington was funded, there existed virtually no federal monies for the support of pure science, a situation that continued to exist until the end of World War II. The first scientists that Carnegie supported here thus had very privileged positions, which they used to make Cold Spring Harbor one of the world's leading centers for genetics, a situation that still existed when I first came here in 1948 as a graduate student of Salvador Luria. Soon afterward, government monies for support of pure science rapidly grew, and by the early 1960s, private universities and research institutions no longer required large endowments to play important roles in science. Even more important for an institution's scientific future was the entrepreneurial spirit that sought out government funds not only for direct research support, but also for construction grants that led to major increases in the facilities available to do high-level science.

The 1962 decision by the Carnegie Institution of Washington to close down its Genetics Department at Cold Spring Harbor Laboratory thus did not immediately threaten the perpetuation of science here. As long as excellent scientists chose to work here, government monies were available to cover not only their research costs, but also their salaries. But it was then more difficult to foresee that Cold Spring Harbor could retain its commanding presence in the field of genetics which had come to it by having on its staff scientists such as Alfred Hershey and Barbara McClintock. Recruiting future intellectual giants would be much more difficult than it had been in the Carnegie years, since the better young molecular geneticists would inevitably be tempted to start their scientific careers in institutions that had federal, state, or endowment funds to cover their salaries. The first several years following the Carnegie pullout were thus rocky years for Cold Spring Harbor Laboratory, and great doubts existed among even its closest supporters as to whether it had a future worth saving.

When I became the director in 1968, I decided to give the Laboratory more stability by focusing its research on the genetic basis of cancer, a field that had long intrigued me dating back to my beginning days as a scientist in Salvador Luria's laboratory at Indiana University. In particular, I wanted to attack cancer through the study of several small animal viruses that were capable of changing normal cells into their cancerous equivalents. I had known how to think in terms of viruses ever since my Ph.D. research, and increasingly, I wanted to follow up my 1959 hunch that tumor viruses possessed genes that upset the normal regulatory pathways which kept cells from multiplying out of control. Happily, there was no shortage of National Cancer Institute monies for such research, and we obtained a sizable grant that allowed me to recruit several very talented young scientists who had already begun research on tumor viruses. Here, Cold Spring Harbor had the advantage in that attacking cancer was not yet a major objective of most of our better intellectual competitors who remained absorbed in working out how genes function in the more simple bacterial systems. We thus stood out as one of few intellectual centers that was moving DNA-based thinking into the world of cancer. Led by Joe Sambrook, whom I recruited from tumor virus research at the then newly formed Salk Institute, this effort quickly yielded results and Cold Spring Harbor soon again displayed the intellectual eclat that dominated the 20-year era led by Miraslov Demerec which had begun in 1941.

Our full potential as a center for biology, however, needed more than the abundance of targeted federal cancer monies. As the director, I needed unrestricted monies so that I could staff other areas of research both through the creation of new research facilities and through the recruitment of new staff members. This freedom to extend our intellectual horizons happily came soon when suddenly in 1973 our neighbor Charles S. Robertson conveyed to us an extraordinary 8 million dollar gift to endow the Robertson Research Fund. From its inception, we decided to focus this fund virtually exclusively toward ensuring the success of the careers of our younger scientists as opposed to providing tenure-like support for those whose research had already taken off. Even though this decision has meant an inevitable loss of key scientists in the 35–40-age bracket to more secure positions at major universities, in the long term, we have benefited from the renewed vigor brought to us by their more youthful replacements.

For almost 10 years, the availability of Robertson funds meant that as director I had the freedom to focus on the doing of science as opposed to worrying about how to fund it. But by 1981, as a result of the newly developed recombinant DNA procedures, the pace of our science suddenly quickened, with it becoming more apparent each year that DNA methodologies were increasingly going to domi-

nate more and more of biology. As a premier institution, we had to accomplish each year even more good science just to stay intellectually even with our better competing institutions. Luckily, the emergence of the first gene cloning companies encouraged moves toward biotechnology by many major industrial companies, and we greatly benefited in the mid 1980s from short-term partnerships with Pioneer Hybrid International, Monsanto, and Exxon. On our side, we helped train their scientists for the biotechnology future while they provided the funds needed to let us move into new areas of recombinant DNA research at full speed. Similar agreements with other companies were made by many other leading institutions, and we began to witness the transformation of the more than 30-year-old bipartite academic-governmental partnership into a troika of academia, government, and industry partners. Crucial to the emergence of this new triumvirate was the 1980 Bayh-Dole legislation, which allowed academic institutions to hold the commercialization rights to their federally funded research. Before this bill was passed, these rights were held by the federal government itself, thereby greatly diminishing the incentives for academic institutions to find industrial partners for their discoveries. Under this new law, academic institutions became free to make deals that gave industrial partners rights to the commercial development of research that had been partially funded with government monies.

In witnessing the emergence of industrial monies as major factors in today's biomedical sciences, we should make clear that this change has not been the result of a real decrease in government support. Instead, the actual driving force is the increased number of highly qualified scientists applying for federal grants. As a result, not only has the size of individual federal grants been going down, but also more and more good grant applications are at best delayed in their funding and at worst turned down. In the past, young scientists who were successful during their postdoctoral period could realistically expect to be funded as soon as they commenced their first academic appointment. Now such individuals have more uncertain futures unless their academic institutions can provide interim funding until their first grants are approved. Until very recently, most high-quality institutions thought that they could effectively house first-class science as long as they provided the necessary research facilities. Today, however, we are entering an era in which to maintain high-quality science on university campuses or within nonprofit research laboratories, these respective institutions must have substantial, internally controlled funds to plug the increasing leaks in our research fabric.

The sums now required are far beyond those that previously could be obtained from private individuals, who in the past have largely been approached for funds to build new buildings or to renovate out-of-date facilities into virtually new laboratory space. Nonetheless, we are bound to increasingly witness more solicitation of private citizens for direct support of science, particularly for those fields such as cancer research where the general public has a strong vested interest in success. Correspondingly, we have also become more dependent on help from the foundation world. But unfortunately, there exist relatively few sizable foundations that are still strongly oriented toward basic science, and, to our dismay, we are witnessing the increasing tendency of peer-review groups to fund most scientists at the same level, independent of whether there are large differences in their relative potential for true innovation. Thus, those science-dominated foundations that do exist, such as the Keck, Markey, Mathers, and Sloan, are finding themselves more and more called upon to maintain the high intellectual goals of our major research institutions.

Almost paradoxically, it may be the elite research-dominated institutions that

have their way of life most threatened by these years of increasing fiscal stringency. Until recently, their major scientists routinely received the multiple research grants needed to support their 10–30-member research groups that have become facts of life for many leaders of this recombinant-DNA-dominated research era. That the largest of such research groups are cost-effective, however, is far from clear, and the money consumed might be more wisely expended if distributed to two independent research groups. But even granted that such excesses do exist, there is no doubt that many of our best scientists need multiple grants to generate the science that our nations expect from them. Vital help for a small fraction of our best scientists now comes from the Howard Hughes Medical Institute, which provides major support for some 200 highly talented structural biologists, molecular geneticists, cell biologists, neurobiologists, and immunologists. But for every well-supported Hughes investigator, there exist some 5–10 other scientists of equal future potential whose productivity remains hostage to the effective crapshoot atmosphere now pervading peer-review group decisions. Fortunately, an increasing number of foundations, such as the Pew Charitable Trusts and the McKnight Foundation, provide career development awards to help highly talented younger scientists get started. But once such awards have expired, their research could easily falter even when their objectives don't deviate too much from conventional wisdom.

So those of us who oversee our major research-oriented institutions are more and more becoming engaged in seeking large-scale industrial funds to keep our science moving forward. But in seeking much more sizable help than we have ever asked for before from the pharmaceutical and biotechnology worlds, we will be asking for more money than can fit under the rubric of charitable contributions. Such transfers of money to us must in return make commercial sense to industry by providing research that otherwise would be carried out in the companies themselves. Conceivably, by placing such research in university-like atmospheres, it can be done faster and cost less than if industries themselves carried out these tasks. Until recently, in general, neither the academic nor the commercial worlds thought that such arrangements were good for either side. Too many years, if not decades, might pass, for example, before the pure research carried out at institutions like Cold Spring Harbor would ever affect the market place. Moreover, those companies that could have so funded us might not be the right ones to develop our prospective products commercially. Today, however, many academic research objectives are indistinguishable from what industry sees it has to accomplish. For example, both worlds wish to know more about how life-threatening cancers develop. Conceivably, such research has a better chance of success if carried out in more academic atmospheres where research goes on all through the night and where weekends are indistinguishable from ordinary days of work.

The pharmaceutical and biotechnology industries now want such partnerships, although it is far from clear that the sums of money they can devote to academic-sited research will ever be even close to the amount needed to correct the deep malaise developing throughout the biomedical world of our country. Enough industrial funds are available, however, to stabilize fiscally a few key institutions, as shown by the recent proposed agreement between the Scripps Research Institute of La Jolla and the Swiss pharmaceutical giant Sandoz. To obtain the right of first refusal for all discoveries made at Scripps, Sandoz was willing to provide 30 million dollars a year to Scripps, whose current budget for science is now approaching 90 million dollars, much of that coming from federal research grants. Key to Sandoz's interest in Scripps was not only the general excellence of

its wide-ranging program in biomedical research, but also, in particular, its recently developed School of Chemistry and its companion drug-discovery group.

The breathtaking size of this proposed agreement, announced early this year, soon brought into the public gaze the question of how deals between industry and academia should be structured, particularly as to whether the proposed deal was for the public good as well as for that of Scripps and Sandoz. Among the concerns raised was whether the presence of Sandoz members on the Scripps Board of Trustees would effectively give Sandoz control over one of our nation's leading research institutions. Equally troubling to many was the possibility that this agreement, if allowed to go forward, would lead other major institutions to similar pacts, thereby making many of the choice fruits of our publicly supported research unavailable to competitive bidding by newly emerging biotechnology companies. By now both NIH and congressional voices have expressed their displeasure, and it is likely that the arrangement will be revised so that Sandoz has less apparent control over Scripps' future and, in particular, its intellectual property rights.

If the finally revised and governmentally smiled upon deal still has the mega-dollar properties guaranteed to insulate Scripps from the financial restraint so apparent elsewhere, their scientists may soon find that they have struck a Faustian-like pact. The knowledge that it is cash-rich will not escape the notice of the NIH peer-review group members, who will invariably come from institutions under siege. Members of its talented Chemistry School are likely to be the first to experience standing last among their peers as priorities are made for the distribution of scarce monies. And even those scientists distant from the world of drug discovery are likely to find themselves disadvantaged when applying for funds. They will belong to an institution that clearly has more than enough funds to tide over grant delays, particularly if it restricts further growth of its facilities and uses restraint in setting scientific salaries. Scripps thus could find itself progressively more dependent on Sandoz support and have to move their research progress increasingly in the directions that are attractive to its industrial partner.

So if a Scripps-Sandoz-like deal were to be offered to Cold Spring Harbor Laboratory, it might not be in our long-term interests to accept it. Much better would be the arranging of much more restricted agreements with a number of companies, each targeted to the needs of specific staff members who have common interests with their specific corporate partner. Thus, while individuals of our staff might have the good fortune of being research-money-rich, their good fortune need not handicap those of our staff whose research is unlikely to appeal to industry and so badly need NIH and/or NSF support. As implied earlier, these latter scientists are likely to be under constant anxiety over the foreseeable future, and we must take on the institutional responsibility to find the funds that will allow their careers to blossom while with us. In this way, we can remain a laboratory that has important, long-term basic research objectives like how our brain functions to let us perceive, memorize, and think.

Only with time will we know whether the acceptance of more and more corporate support will seriously move us away from seeking profound explanations for fundamental cellular and molecular processes that underlie living systems. Certainly, if we move too far, we will face demise as an intellectually alive institution. But at the same time, we must not fall into the all too often academic trap of believing that because a research objective is purer than pure, it is more worthwhile to do than projects with obvious practical objectives. Instead, there are often very good reasons to seek out research goals that are simultaneously

pure as well as applied and so clearly fundable. This was until recently most certainly the case for cancer research, where the uniqueness of cancer cells can only be understood when related to the comparative behavior of normal cells. Unfortunately, during the last several years, the increasing number of scientists interested in cancer genes began to put pressures on the money available for so-called untargeted cancer research. But those competent scientists interested specifically say in breast cancer still have no funding dilemmas.

There should thus be no undue pessimism about the immediate future of Cold Spring Harbor Laboratory as long as we keep a strong sense of what both we and the public want from science. Increasing connections to industry need not hurt us as long as the companies we work with also see the need to understand life ever deeper at the molecular and cellular levels. So institutions that have the apparent good fortune to remain totally governmentally funded, such as the MRC-supported Laboratory of Molecular Biology at Cambridge or the vast intramural program of NIH at Bethesda, will not necessarily do more inspired science than those of us who have multiple masters controlling our fates. Conceivably, they may suffer more from the lack of need to constantly reappraise their courses than we will suffer from the anxiety that comes from simultaneously displaying both pure and applied faces.

Our next years obviously will not be carefree, but neither were the great years of our past.

HIGHLIGHTS OF THE YEAR

An Exciting Year Scientifically

This has been an active and exciting year for Cold Spring Harbor science. New developments in the biology of the cell cycle, the cytoskeleton, aging, plant biology, and neurobiology convey some of the diversity of Laboratory research in 1992.

David Helfman and Jim Lees-Miller, in collaboration with Trina Schroer of Johns Hopkins University, found a major piece of an intracellular "motor." This protein, related to the molecule actin, which helps muscles contract, is called actin-RPV. It appears to be involved in moving organelles around the inside of the cell. Actin-RPV may therefore be an important piece of the machinery that ensures a molecule is in the right place at the right time. In addition, because it is associated with the spindle poles that form during mitosis, actin-RPV may turn out to play a role in cell division.

Staff investigator Xiaodong Cheng has solved the structure of the first DNA methylase. Working with enzymes from the bacterium *Hemophilus*, Xiaodong solved the structure to a resolution of 2.5 Å. In bacteria, methylases act in concert with restriction enzymes to regulate the cutting of DNA. In eukaryotes, however, they serve a more complex function as part of a group of enzymes responsible for DNA replication and in the regulation of gene expression.

Grisha Enikolopov works on *Science* magazine's "Molecule of the Year." Nitric oxide (NO) is a gas that functions in transmitting signals between nerve cells. NO seems to act in concert with other messengers, such as calcium, to enhance

their effects. It thus acts as a sort of molecular volume knob, modulating the impact of a given neurotransmitter. NO first made science headlines because it is a "retrograde" messenger. It exerts its effects backward with respect to the electrical nerve signal, from the receiving cell to the sending cell. NO thus closes a feedback loop; the sending cell signals to a receiver, and the receiver, via NO, "responds" with a signal back to the sender that changes gene transcription.

Postdoc Steven Bell and senior staff scientist Bruce Stillman discovered what some refer to as the "Holy Grail of DNA replication." The grail in this case is a cluster of proteins that bind to a specific site on the DNA to begin replication. When a cell divides, complete replication of the DNA must occur to ensure that each new "daughter cell" gets a full set of genes. The finding is important because it links research on the signals that trigger cell division with research on how cell division actually happens.

Senior staff investigator Carol Greider and colleagues at McMaster University found a new potential link between the chromosomes and cancer. Telomeres, the ends of chromosomes, consist of many repeats of a simple DNA base sequence. In 1991, Greider along with Bruce Futcher showed that telomeres shorten with age. This is the result of an enzyme Greider discovered called telomerase, which chops off telomere sequences each time a cell divides. In this latest work, she showed that telomerase is not active in cultured cancer cells that become "immortal"; thus, the telomeres of immortal cells do not shorten like those of normal cells. The next question in this research is whether telomere shortening is a cause or an effect of immortalization.

Mike Gilman's laboratory developed a "cell-free" system for studying signal transduction, the mechanism of bringing chemical signals from the outside of the cell, such as hormonal signals, into the cell and into the nucleus, where they change the cell's behavior. What Gilman has done in essence is to reproduce certain aspects of signal transduction in a test tube. This allows a more direct examination of the mechanisms of signal transduction. From a practical standpoint, the system may provide an exceptionally simple assay for novel compounds that either interfere with or augment signal transduction; these could have significant therapeutic potential.

Proteins called G_1 cyclins are key controllers of cell division in yeast. Bruce Futcher and David Beach looked for similar proteins in human cells and found one they called cyclin D. Importantly, this cyclin turned out to be aberrantly expressed in many cancers. These findings indicate that (1) the most basic controls on cell division are conserved from yeast to humans and (2) at least some cancers may result from disturbances of the cyclins that control cell division.

Nikolai Lisitsyn, Natalya Lisitsyn, and Michael Wigler have developed a rapid new way to identify disease-related genes in human DNA. Called the "representational difference method," this technique enables researchers to compare the DNA of normal individuals with the DNA of afflicted individuals to isolate the DNA regions gained or lost in disease carriers. Although a similar technique had been developed for bacteriophage and yeast, until now, no one had succeeded in using it on human DNA. Wigler and Lisitsyn's method uses some clever applications of the polymerase chain reaction to circumvent problems posed by the large size of the human genome. Preliminary data indicate that this technique may provide a powerful way to identify genes underlying hereditary diseases and certain types of mutations involved in cancer.

Using one of the transposable elements first discovered by Barbara McClintock, senior staff investigator Tom Peterson is firmly rooted in classical maize genetics. Modern techniques of molecular biology, however, allow Peter-

Dr. Michael Brown at Dorcas Cummings Lecture

son and his group to explain in molecular detail how the *Ac* element jumps in and out of the maize *P* gene. In addition, the *Ac* element creates "mutations" within the *P* gene; each *Ac* insertion site produces a different pigmentation pattern in the mature ear. By comparing these insertion sites with the resulting pigment patterns, Peterson learns about the function of different regions of the gene.

Symposium

This year marked the 57th Cold Spring Harbor Symposium on Quantitative Biology. The topic was "The Cell Surface," and the meeting was organized by Richard Axel of Columbia University, Corey Goodman of Berkeley, Richard Hynes of MIT, and our own Bruce Stillman. The diversity of research interests represented by this group of organizers reflects the breadth of the topic for this year's meeting. The Symposium featured talks on a wide range of subjects, including immunology, neurobiology, developmental biology, and cellular communication. This year's Dorcas Cummings Lecture was given by Michael Brown, M.D., of Southwestern Medical School in Dallas. Presented on the Sunday of the Symposium, the Dorcas Cummings lecture features a prominent scientist speaking to the public, rather than to an audience of specialists. Dr. Brown, who shared the 1985 Nobel prize in Physiology or Medicine with his collaborator Joe Goldstein, discussed that fatty molecule so often talked about in the popular press: cholesterol. In his lively talk, Brown explained the difference between HDLs and LDLs, what cholesterol counts really mean, and how much genes may influence the risk of heart disease.

Banbury Meetings Pushing the Envelope

The Lloyd Harbor estate donated to the Laboratory in 1976 by Charles Robertson is the setting for an ongoing series of small, in-depth conferences assembled by Director Jan Witkowski. These meetings, typically 3 days in length and attended by about 40 people, run the gamut from highly technical presentations of new

laboratory results and techniques to scientific topics with a strong social relevance. Over the course of a year, Banbury attendees include leading scientists, congressional staffers, corporate heads of biotechnology corporations, and journalists. This year's schedule contained meetings on the control of gene expression in the AIDS virus and mechanisms of neuronal survival, as well as workshops on the commercialization of biology, the human genome, and the impact of indirect costs on independent research institutions. The Banbury meetings continue to push the envelope of knowledge within and between pressing topics in biomedical research.

Baring Brothers Executive Meeting

A highlight of the annual Banbury schedule is the meeting sponsored by Baring Brothers & Co., Ltd., the celebrated English merchant bank. The conference is founded on the idea that the industries affected by science benefit from first-hand interactions with the people, ideas, and techniques of science. Each October, business executives from the biotechnology, pharmaceutical, and financial communities convene to hear leading researchers discuss their latest findings. This year's topic was aging; the conference featured talks on the longevity revolution, neurodegenerative diseases, and prions, the controversial disease-causing mini-proteins. In addition, Mark Bloom and David Micklos of the DNA Learning Center led the executives through a laboratory exercise in DNA fingerprinting.

A Vigorous Board of Trustees

Dr. Barney Clarkson

Our board of trustees helps shape Laboratory decisions on a wide range of issues, including new construction, hiring of scientists, and raising money. Of the 30 trustees, 10 are working scientists; the remaining 20 public members bring expertise in business, the law, or public service. They live primarily on Long Island or in New York City and join us because of their interest in promoting biomedical research. Without a strong board of trustees, the Laboratory would not be the success it is. Each member is limited to two 3-year terms on the board, and each year some members are required to relinquish their seats. This year saw the retirement of six board members, including two officers. We have a brand new set of officers and six new members who I am sure will be valuable additions to our board.

Special mention must be given to Barney Clarkson, M.D., our retiring chairman. Barney and I have worked together for many years, and his advice has guided me and the Laboratory through many mazes. Barney is a valued friend as well, and I hope that he will continue as a member of the Laboratory community in his new role as honorary trustee. In recognition of Barney's service to the Laboratory over the years, we dedicated the new air-conditioned downstairs dining room in Blackford Hall the Clarkson Dining Room.

Vice chairman Taggart Whipple also retired at our November trustees meeting. Enough cannot be said about Tag. He was a long-time friend and adviser who helped with, among other things, many legal aspects of running an institution such as this. Sadly, shortly after the November meeting, Tag passed away. Everyone who knew him will miss his sharp mind and kind heart.

Bill Everdell retired from our board as well. He has been an important and generous supporter of the Laboratory. At our November meeting, Bill was presented with a Steuben crystal vase etched with his name and the CSHL logo. Our

other retiring trustees were Tom Maniatis, David Sabatini, and Jonathan Warner. Tom is best known in Laboratory circles as the author of the lab-bench bible *Molecular Cloning*. He was a scientist at Cold Spring Harbor Laboratory from 1974 to 1976, and from 1985 to 1992, he was chairman of Harvard's Department of Biochemistry and Molecular Biology. While on our board, Tom served on the Banbury Meetings Program committee and as chairman of the Tenure and Appointments committee. David Sabatini, chairman of the Department of Cell Biology at New York University, also served on the Banbury and Tenure and Appointments committees and on the DNA Learning Center committee as well. Jonathan Warner, director of the Division of Biological Sciences and chairman of the Department of Cell Biology at the Albert Einstein College of Medicine, first came to the Laboratory in 1963 to attend the Symposium on Synthesis and Structure of Macromolecules. He was an active member of the Banbury committee.

Our new members are an excellent group of scientific and business leaders. Joan Steitz is a Howard Hughes Medical Institute Investigator and the Henry Ford II Professor of Molecular Biophysics at Yale. She is a leading researcher in the field of RNA processing. J. Anthony Movshon is an American-born, British-trained neuroscientist. Director of New York University's Center for Neural Science and a Howard Hughes Medical Investigator, Tony studies the visual system. We look forward especially to his input on our neuroscience program. Don Wiley, also a Howard Hughes Medical Investigator, became chairman of Harvard's Department of Biochemistry and Molecular Biology in 1992 and is a member of the National Academy of Sciences.

Edwin Marks studied at Princeton and the U.S. Military Academy, where he received a B.S. in engineering. Since 1961, he has been president of the New York investment firm Carl Marks & Co., founded by his father. He is also a member of a number of cultural, educational, and charitable organizations, including the Lincoln Center for the Performing Arts and the Chief Executives Forum. John J. Phelan, Jr. is a retired chairman and Chief Executive Officer of the New York Stock Exchange. A magna cum laude graduate of Adelphi University, he holds honorary doctor of laws degrees from seven universities. He is a member of the Council on Foreign Relations, president of the International Federation of Stock Exchanges, and a member of numerous corporate, charitable, and philanthropic boards. Thomas A. Saunders III is a graduate of the Virginia Military Institute and the University of Virginia's Colgate Darden Graduate School of Business Administration. A 22-year veteran of the New York firm of Morgan Stanley, Saunders is now a general partner of Saunders, Garonzik & Karp. He and his wife Jordan live in Locust Valley.

This large turnover in our board of trustees has led to a new group of officers. The new chairman of the board is David Luke, who so ably chaired the Second Century Campaign. His vice chairman is Mary Lindsay, a long-time and enthusiastic Laboratory supporter. John Reese has taken over the job of treasurer. Wendy Hatch is now serving as secretary of the board, with Townsend Knight serving as assistant secretary.

Second Century Campaign Concludes

Mounted in preparation for the Laboratory's 100th birthday in 1990, the Second Century Campaign drew to a close at the end of 1992. Under the steady hand of chairman David Luke, the Campaign met and surpassed what initially was thought to be an optimistic goal. Originally set at $40 million, the goal of the

Second Century Campaign volunteers David Luke, Wendy Vander Poel Hatch, Townsend J. Knight, and George W. Cutting

Campaign was raised to $44 million. By the end of this year, $51 million had been raised to support Laboratory programs. We are grateful to David Luke and the large cadre of volunteers who made this campaign so successful.

Robertson Research Fund

The Robertson Research Fund, established in 1973, is a mainstay of the Laboratory's endowment. It supports plant genetics and other research, contributes to meetings, courses, and travel and relocation expenses for Laboratory scientists, and provides backup support for underfunded research projects and meetings. The Fund supports the Outstanding Junior Fellow Award, which this year went to David Barford, a crystallographer who studies a class of enzymes called protein phosphatases. The Fund also supported several postdoctoral fellows and staff scientists.

Major Gifts

Without the support of generous individuals and corporations, the Laboratory could not continue to carry out basic research. In 1992, we received substantial gifts of many types that will support scientists, help provide researchers with modern facilities, and contribute to DNA education of children and teachers. Our infrastructure fund received pledges totaling $650,000—a crucial form of support that is not always easy to find. We received major new gifts for infrastructure from the Baxter Foundation, Marion Merrell Dow Inc., and Pfizer Inc. The Westvaco Corporation this year doubled their gift from 1991.

The cancer fund supports all aspects of cancer biology research at the Laboratory, still the backbone of Cold Spring Harbor research. Our cancer fund received pledges totaling nearly $2.0 million in 1992. Major gifts received from Eleanore and William Everdell, Robert Gardner, the estate of Charles Leach II, George and Mary Lindsay, Thomas and Jordan Saunders, the Pritchard Trust, the Farish Fund, J.P. Morgan & Co., the Marks Family Foundation, the Bodman Foundation, the Achelis Foundation, and the Hyde and Watson Foundation will

help ensure progress in our basic understanding of cancer. Our education fund received major support at a crucial time from Cablevision and the Harris Trust, totaling $300,000. This fund supports the DNA Learning Center, which is growing rapidly and in 1993 will expand to include a new theater and more laboratory and exhibit space.

We received four major contributions to our endowment, which is now stronger than ever. The W.M. Keck Foundation pledged $1.5 million to found the Keck Chair in Structural Biology. $1.5 million was received from the estate of the late Maxine Harrison to establish a chair in memory of her mother Alle Davis Harrison for the study of molecular neuroscience. The Fort Hill Foundation, established by the family of William J. Matheson, successor to Eugene Blackford as president of the Biological Laboratory in 1904, pledged $500,000 to found the William Matheson Chair. The Banbury Fund, administered by the Robertson family, added to the endowment that supports the Robertson Chair for Neuroscience. As a contribution to Planned Giving, the four daughters of Sara D. Redmond donated a house previously owned by the family in Oyster Bay.

Finally, the Annual Fund reached an all-time high total of $560,334 in 1992. The Annual Fund comprises gifts intended to be used in the year they are given and goes to support many aspects of the Laboratory, including startup money for young investigators, support for postdoctoral fellows, renovations, and equipment.

Blackford Hall Remodeling Finished

In 1992, renovations to Blackford Hall were completed. Bill Grover and Jim Chil dress of Centerbrook Architects, with Jack Richards' able staff, have executed an architectural version of the proverbial 50 clowns in a circus car. They managed to double Blackford's dining capacity, enlarge the bar, expand the deck, add a

J.D. Watson, Dr. Franziska Racker, and Ann Racker Costello

Racker Reading Room in renovated Blackford Hall

game room, modernize the kitchen, and provide a formal dining room, while only minimally changing the outward appearance of the building. The aspect from Bungtown Road is scarcely changed, and the harbor side of the building seems merely to have the area under the deck enclosed. Updating the 1907 building enables us to accommodate the ever-swelling number of meetings attendees. Blackford can now serve 400 people at one sitting, which, not coincidentally, is about how many attended our larger meetings last year.

Although much modernized, the building retains its historical feel; old-timers returning to Blackford will still recognize it, inside and out. Thanks for this go not only to Centerbrook, but also to Liz Watson and Laura Hyman, who arranged the decor, particularly of the Racker Reading Room (formerly the fireplace room). Four oversized "mission" style chairs—two originals bought from A&S in 1906 and two replicas—preside over the room, and portraits of scientists who worked and lived here in the 1940s, 1950s, and 1960s hang on the walls. The portraits were drawn or painted by Ef Racker, a scientist and "artist-in-residence" here in the late 1940s and early 1950s, and were generously donated by Franzie Racker. Other Rackers grace the two upstairs dining rooms, which were named the Mayr Dining Room, for Ernst Mayr, and the Hotchkiss Dining Room, for Rollin Hotchkiss. The new downstairs dining room, which is the only room in Blackford that is air-conditioned, was recently named the Clarkson Dining Room, for Dr. Bayard Clarkson, our just-retired chairman of the board of trustees.

McClintock Renovation Continues Apace

Renovation of McClintock laboratory will be completed in 1993. The most dramatic change to the 1914 structure will be the third story being added to the top. With lots of glass and a copper roof, the addition should blend with the original feel of the building while providing much-needed space for offices and

conference rooms. Below, the exterior is intact, but the former "animal house" will receive an all-new circulatory system and digestive tract, bringing it up to modern standards for a molecular biology laboratory. Once completed, David Beach will set up shop on the second floor and continue his investigations into the genes and proteins of the cell cycle. Dick McCombie will move to the first floor and set up a gene-sequencing laboratory there. Working in collaboration with Beach and Tom Marr, McCombie is mounting a "pombe genome project," which seeks to sequence the entire genome of the model yeast *Schizosaccharomyces pombe*.

DNA Learning Center

The DNA Learning Center greatly extended its reach this year in its continuing effort to bring DNA education to the public. A major step was the hiring of Robert Willis as special programs manager. Bob is responsible for teaching in the *Bio2000* Laboratory, ordering supplies and other aspects of keeping the teaching program up and running. One of his goals is to recruit disadvantaged minority students from the New York City area to come to the Learning Center to participate in laboratory workshops. Bob is also involved with the *Fun With DNA* summer workshops. Three of these workshops this coming summer will be targeted especially at minority students.

Late in the year, the Learning Center staff began dismantling the Smithsonian Institution "Search for Life" exhibit that had been up since the Learning Center opened in 1987, in order to make space for a new 100-seat auditorium. The DNA Learning Center will provide the venue for Cablevision's forthcoming multimedia presentation on Long Island, which in turn will feature a segment on Cold Spring Harbor Laboratory. In addition to using the auditorium for its own presentations, the Learning Center will make the new hall available to other small museums in the area.

The Learning Center's ground-breaking teaching programs reached new corners of the globe this year, with collaborations with new science museums in Sardinia, Italy, and Svalof, Sweden. In these collaborations, the Learning Center staff helped set up new DNA exhibits and had an opportunity to field test experimental interactive computer exhibits. These include "DNA Detective," which takes students through the process of DNA fingerprinting and gives them a chance to solve a crime using DNA evidence.

Carol Greider Honored for Research on Aging

Senior staff investigator Carol Greider was named the first recipient of the Allied Signal Outstanding Project Award for Biomedical Research. Carol's research proposal was selected from a pool of more than 50 applicants in the field of research on aging. The award consists of a check for $200,000, which goes to the Laboratory in support of Carol's research. Allied Signal is an advanced technology company with businesses in aerospace, automotive products, and engineered materials. The research, carried out jointly by Carol and her colleague Calvin Harley, focuses on telomeres, the ends of chromosomes. Carol previously showed that telomeres shorten with age. An enzyme called telomerase is responsible for the shortening; each time a cell divides, telomerase snips off a portion of the telomere region. Although it is not yet known whether aging causes telomere shortening or vice versa, shrinking telomeres may serve as a molecular clock, indicating the age of the cell.

Carol Greider

Jacek Skowronski

Jacek Skowronski Wins Johnson & Johnson Award

Staff investigator Jacek Skowronski was awarded a 3-year, $270,000 grant from Johnson & Johnson. The award supports Jacek's work on the AIDS virus and was accompanied by a luncheon and presentation in his honor. Jacek works on the gene *nef*, an AIDS virus gene that seems to play a role in disrupting the immune system of the host animal. To study this gene, he inserts it into the immune cells of mice, making a lineage of transgenic animals that have this single AIDS virus gene only in the cells where the gene acts.

CSHL Press

The Cold Spring Harbor Laboratory Press continued to grow this year, publishing 15 new books in 1992. A showpiece of the 1992 catalog is *The Dynamic Genome*, a Festschrift honoring Barbara McClintock's 90th birthday in June. Edited by Nina Fedoroff and David Botstein, this collection of essays includes hard science, history, reminiscence, and anecdote, putting Barbara's life and ideas in context with the development of modern genetics. The Press also reissued the 1966 classic Festschrift for Max Delbrück, *Phage and the Origins of Molecular Biology*, edited by John Cairns, James Watson, and Gunther Stent. The new edition is expanded to include Stent's obituary of Delbrück, two commentaries, reprinted from *Scientific American* and *Science*, on issues raised in the book, and a new preface by John Cairns. Other new titles this year include *The Cell Cycle*, the proceedings of Symposium 56; *A Short Course in Bacterial Genetics*, an instantly successful laboratory manual; *DNA on Trial*, now becoming popular among lawyers who need to grasp the significance of genetic evidence for the courtroom; and *Transcriptional Regulation*, a major monograph in the Cold Spring Harbor tradition, edited by Keith Yamamoto and Steve McKnight.

One of the bread-and-butter titles for the Press, the second edition of *Molecular Cloning: A Laboratory Manual,* sold briskly in 1992, with sales surpassing even those of the first edition. *Antibodies: A Laboratory Manual,* written by former Cold Spring Harbor scientist Ed Harlow, also sold extremely well this year. The Press's journals are also doing well. *Genes & Development* increased its circulation and was named for the third year in a row the journal with the highest impact factor in the fields of genetics and developmental biology by the Institute for Scientific Information. Manuscript submissions also increased, making it likely the journal will publish more issues in 1993. *PCR Methods and Applications* completed its rookie year and began its second volume in the black—no mean feat, given today's economic climate in publishing. Increases in advertising sales and manuscript submissions, as well as sponsorship from Perkin-Elmer, will enable the journal to go from four to six issues per year in 1993.

The coming year promises to bring further growth for the Press. Twenty-five new titles are planned, including lab manuals on techniques for fission yeast, plant molecular biology, protein chemistry, and the biology of nerve cells. Several monographs are also planned, including works on RNA, reverse transcriptase, and a definitive reference on *Drosophila* development.

Mikhail Yanovitsky

Special Events

The Laboratory calendar featured a number of unusual lectures and cultural events. Pianist Mikhail Yanovitsky performed a program of Bach, Beethoven, and Schubert. Michael Novacek, vice president and Dean of Science at the American

Drs. Mark Lippman (*left*) and Mary-Claire King (*right*) at Cold Spring Harbor Laboratory seminar on breast cancer

Museum of Natural History, presented an interesting talk on paleontology in Mongolia, complete with slides of new species of fossil dinosaurs and of fossil dinosaur eggs. Breast cancer specialists Mary-Claire King and Mark Lippman returned to give an update on the search for a breast cancer gene. The two scientists also helped allay fears about the infamously high rate of incidence of breast cancer on Long Island. Rounding out the schedule was Ron Davis' Lloyd Harbor Seminar on learning and memory research. The Lloyd Harbor series, held at the Banbury Center, is organized by George Toumanoff for the benefit of Banbury neighbors.

Undergraduate Research Program

Each summer since 1959, approximately 20 undergraduates from around the country and overseas have come to the Laboratory to carry out research projects with our scientists. This year, 23 students participated in the 10-week program, coming from Berkeley, Harvard, Tuskegee, Cambridge, and Glasgow Universities, among others. The students learned new techniques and carried out research projects, working on such topics as learning and memory in *Drosophila*, the maize *P* gene, the *ras* oncogene, protein structure, and pattern recognition in DNA sequences. Each student has a sponsor, with support coming from Baring Brothers & Co., Ltd., the Burroughs Wellcome Fund, Theodore N. Danforth, Hanson Industries, the National Science Foundation, the Robert P. Olney Fund, and the Phillips Petroleum Foundation, Inc.

Partners for the Future

In 1990, the Laboratory extended the idea of the Undergraduate Research Program to high school students through the Partners for the Future program. It has proved successful in attracting some of Long Island's best and brightest high school seniors into the laboratory to give them a taste of cutting-edge molecular biology research. Sponsored by area businesses, students receive a stipend as well as hands-on experience unattainable in a classroom. Five students were selected from 150 Long Island schools to spend 10 hours per week after school, from October to March, under the tutelage of a Cold Spring Harbor mentor. This year's Partners, with their school and mentor, were Tara Adamovich, Garden City High School (Dr. Robert Franza); Jason Altman, Herricks High School (Dr. Elizabeth Moran); Rafaz Hoque, Commack High School (Drs. Tim Tully and

Michael Regulski); Munfarah Hossain, Walt Whitman High School (Dr. Yi Zhong); and Jayson Mystkowski, Kings Park High School (Dr. Venkatesan Sundaresan).

Richard Roberts

Long-term Service

In 1992, three employees completed 20 years of service to the Laboratory. Senior Staff Scientist Richard Roberts, who obtained his Ph.D. in organic chemistry at Sheffield, England, came here after postdoctoral research at Harvard University to lead our efforts in nucleic acid chemistry. Susan Cooper came to the Laboratory from the University of California library system at Irvine to head our library. Today, she ably directs both the Library and the Public Affairs Office. Terri Grodzicker, whose Ph.D. was from Columbia University, came to James Laboratory to work on tumor viruses after postdoctoral research at Harvard Medical School. Now she is Assistant Director for Academic Affairs for the Laboratory as well as editor of our journal *Genes and Development*.

Changes in Scientific Staff

Senior Staff Scientist and Assistant Director for Research Richard Roberts ended a distinguished tenure of research and administration at Cold Spring Harbor this year. Although temporarily still on the rolls quarter-time, Roberts has moved on to New England Bio-Labs, in Beverly, Massachusetts, near Boston, to become director of research. His important scientific contributions here have included leading the world in the discovery of important new restriction enzymes and orchestrating the Cold Spring Harbor role in the seminal discovery of RNA splicing. Rich also played a major role in the day to day life of the Laboratory, in particular through his role as Assistant Director of Research that commenced in 1986. In bringing computers to Cold Spring Harbor, in attracting the Core Facility, and in advising younger colleagues on their grant proposals, Rich was an important part of the growth and success of the Laboratory from 1972 to 1992, and we shall always remain indebted to him for his devotion to our purposes.

Staff investigator Eric Richards has moved on to Washington University's Department of Biology, in St. Louis, where he landed an assistant professorship. He will be continuing his work on chromosome structure. Three staff associates have also left the Laboratory. Tamar Michaeli, from Mike Wigler's lab, is now an assistant professor at the Albert Einstein College of Medicine, in the Bronx. Will Phares, from Winship Herr's lab, has moved to Vienna, to the Sandoz Research Institute, where he is a laboratory leader. Jim Lees-Miller has left David Helfman's lab to take a position in Calgary, Alberta.

New Staff Members

The Laboratory boosted its programs in neuroscience and genome research this year with the hiring of three new staff scientists. Senior staff investigator Richard McCombie comes to us from the National Institutes of Health in Bethesda, Maryland. He is establishing a "pombe genome project" here in collaboration with David Beach and Tom Marr. He will be working on the first floor of the remodeled McClintock laboratory when renovations are completed. Senior staff investigator Alcino Silva arrived from the Massachusetts Institute of Technology this fall. His exciting work with genetic "knockout" mice promises new insights into mammalian learning and memory. Staff investigator Yi Zhong, coming to us from the University of Iowa, further strengthens our research efforts in *Drosophila* learning

and memory. Four visiting scientists joined us this year. Nicholas Muzyczka, of Stony Brook Medical School's Department of Microbiology, is spending a sabbatical in Bruce Stillman's laboratory. Robert W. West, Jr., from the SUNY Health Science Center in Syracuse, is working with Ron Davis. Frederick Schachat, of Duke Medical School, is working in David Helfman's lab. and Roussoudan Bourtchouladze has joined Alcino Silva's lab from the Anokhin Institute of Normal Physiologie, in Moscow.

David Spector

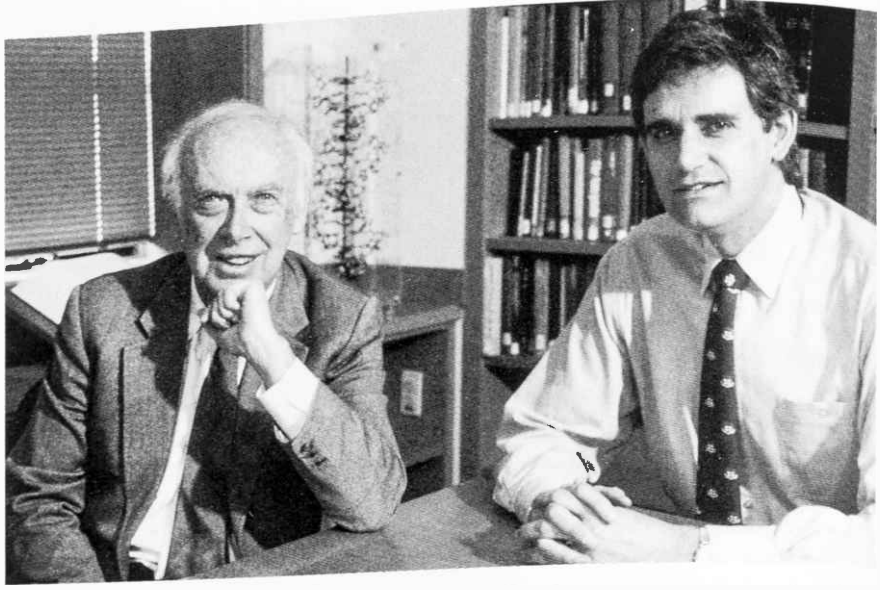
Staff Promotions

With the approval of our Board of Trustees, David Spector was promoted to Senior Staff Scientist in the fall of 1992. David came to us from Baylor in 1985. His research focuses on RNA splicing and nuclear structure. David also runs the Laboratory's electron microscope facility in Cairns lab. Hong Ma was promoted to Senior Staff Investigator. He continues his research on the molecular biology of the model plant *Arabidopsis*. Several scientists became Staff Investigators, including Masafumi Tanaka, Roymarie Ballester, Nikolai Lisitsyn, and Gilbert Morris; Ann Sutton, from Kim Arndt's lab, and Mark Pittenger, from David Helfman's lab, became Staff Associates. Carmelita Bautista was promoted to Research Associate.

Postdoctoral Fellows

A number of Cold Spring Harbor postdoctoral fellows have moved on to other positions and locales. Gary Otto, from Rich Robert's lab, is now a research associate at the Courant Institute of Mathematical Science at New York University. Saulius Kulakauskas, also from Roberts' lab, is now at the INRA in France. Another Roberts, postdoc, Denise Roberts, is now a project leader at Oncogene Science, in Uniondale, New York. Maria Jose-Frenandez Sarabia, a postdoc with Kim Arndt, has moved to Onyx Pharmaceuticals in Richmond, California. Sushma Abraham-Ogram, a postdoc with Betty Moran, is now at Washington State University, in Pullman. Yasuji Rikitake, also from Betty Moran's lab, is now at Medical Environment Co., in Tokyo. Steven Brill, from Bruce Stillman's lab, has taken a job as assistant professor at Rutgers. Also from Stillman's lab, Glenn Bauer has moved to St. Michael's College, in Colchester, Vermont, as assistant professor. Anindya Dutta, also from Bruce Stillman's lab, is now an instructor at Brigham and Women's Hospital, Harvard Medical School. Chiu K. Cheung, from John Anderson's lab, has gone back to school full-time at Touro Law Center. Chitra Kannabiran, a postdoc with Mike Mathews, is now at Rutgers. Mark Kessler, also from Mathews' lab, is now a senior scientist at Biotechnology General in Nes Ziona, Israel. Kwang-Ai Won has left Mike Gilman's lab for the Scripps Research Institute in La Jolla, California. Toru Mizukami, a postdoc with David Beach, is now at the Kyowa Hakko Tokyo Research Laboratories.

Two graduate students working at the Laboratory have moved on to postdoctoral fellowships. Lisa Molz, from David Beach's lab, is now at the Cardiovascular Research Institute at the University of California at San Francisco. Laura Berkowitz, from Mike Gilman's lab, is now at Indiana University's Department of Biology in Bloomingdale. Two other graduate students are pursuing medical degrees. Fred Bunz, from Bruce Stillman's lab, is now at the University at Stony Brook's medical school, and Yuhong Qiu, from Ron Davis' lab, is at the Anderson Cancer Center at the University of Texas, Houston.



J.D. Watson and Bruce Stillman

Appointment of Bruce Stillman as Director to Commence January 1, 1994

This year marks my 25th year as the director, having formally assumed the position in February of 1968 while remaining a professor at Harvard. Then I was not quite 40 years old and still a very active participant in science, running with Wally Gilbert a major laboratory at Harvard and on a first name basis with most of the active players in the world who worked with DNA. After Charles S. Robertson's 1973 gift of the Robertson Research Fund, the Laboratory had the means to bring me on as a full-time director. So in the fall of 1974, my family and I moved into the director's house, which had just been totally renovated, using plans prepared by the noted architect, Charles Moore. Long-called "Airslie", this 1806 wooden dwelling, located above the sand spit at the end of Bungtown Road, has been a perfect home for me and my family, and we have been very privileged to live among the beauty of its great lawns and trees that date from its period as part of the once grand Henry DeForest Estate. Now we will soon leave Airslie, for the time has come for me to step down as director. At 65, I know that I am no longer the active participant in science that the Laboratory needs to oversee its research and recruit the talented young brains that will keep us at the forefront of science. But if I were to stay on as director much longer, I might all too soon lose this clarity of vision.

Happily in Bruce Stillman, who first came to work at Cold Spring Harbor in 1979, we have the perfect future director. This change will formally occur on January 1, 1994, although Bruce has effectively acted in his new role since our February 27, 1993 Board meeting when he was formally selected by the Board of Trustees as the new director.

Bruce, Australian by birth, was born in 1953 and educated at the University of Sydney and the Australian National University where he took his Ph.D. in 1979. From there, he came directly here as a postdoctoral fellow for two years, at the conclusion of which he joined our staff in 1981. Rapidly rising through our ranks, his appointment as a Senior Staff Scientist occurred in 1985. Beginning already as a Ph.D. student, his research has been on DNA replication, initially focusing

on adenovirus DNA. Now regarded as one of the world's leading scientists interested in DNA replication, Bruce most appropriately was elected to be a Fellow of the Royal Society in March of this year.

Bruce's high intelligence combined with his common sense approach to life and the ease with which he works effectively on behalf of others here led to his appointment as Assistant Director in 1990. In that capacity, he has shown great leadership capabilities, and everyone here looks forward to assisting him in his new role.

Creation of the Position of President

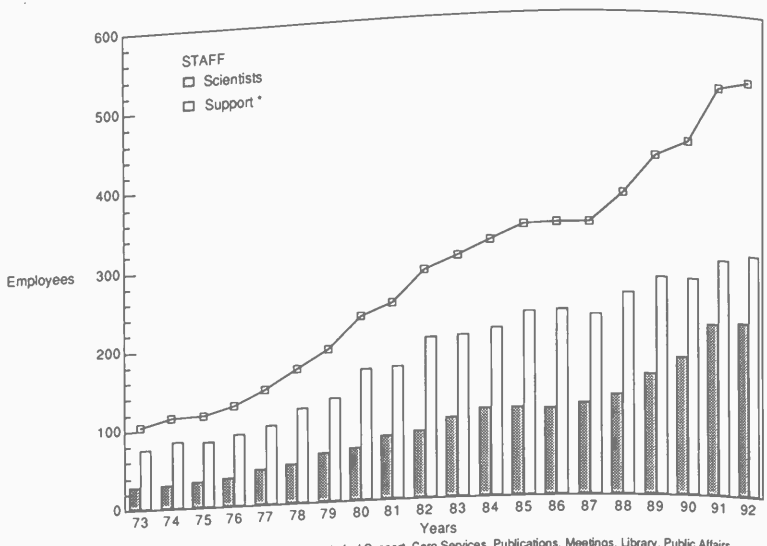
Happily for me, our Board of Trustees at the same time created the new position of President that will continue to allow me to retain a major role at the Laboratory. As President, I will report to the Board of Trustees, and in addition to overseeing the Laboratory's ever-continuing quest for funds, I will have reporting to me the directors of the DNA Learning Center, the Cold Spring Harbor Laboratory Press, and the Banbury Center, with Bruce taking on the additional responsibility for our advanced courses and the meetings held in Grace Auditorium. To let us remain on the Laboratory grounds, the Trustees have authorized the construction of a new President's House that will be built on the site where the Henry DeForest house stood until 1943. Centerbrook of Essex, Connecticut, the distinguished architectural firm that the Laboratory has used for all its major building projects since 1973, has provided plans for a beautiful house into which my wife and I will move in the summer of 1994.

I have every reason still to look forward to the future.

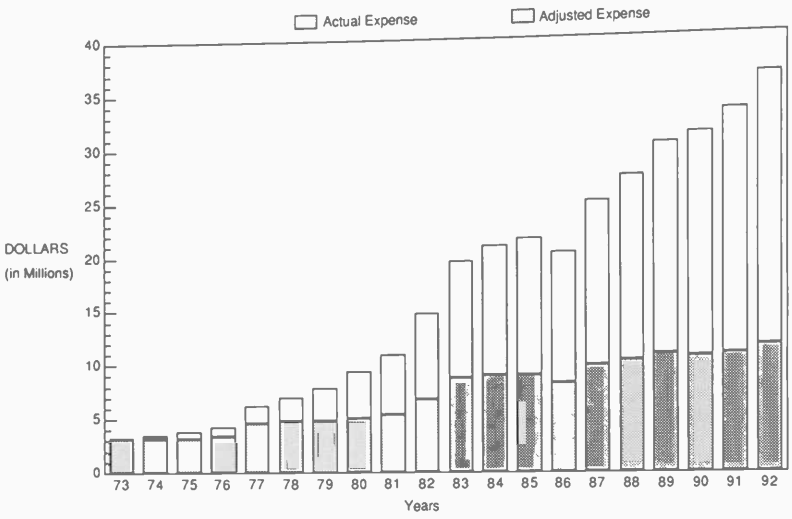
August 4, 1993

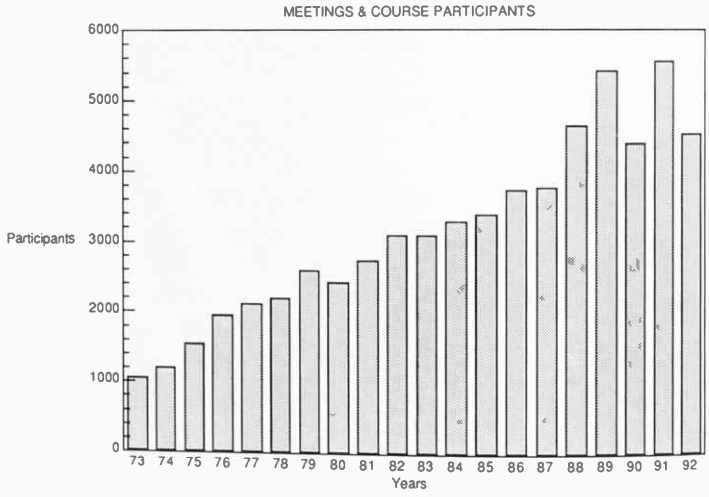
James D. Watson

DEPARTMENTAL REPORTS



* Consists of Full-time and Part-time Technical Support, Core Services, Publications, Meetings, Library, Public Affairs, Buildings and Grounds, Administrative Personnel, Banbury Center, and DNA Learning Center





ADMINISTRATION

1992 was a year of extraordinary science at the Laboratory, marked also by concern that there may not be adequate funding from traditional sources for the unprecedented opportunities ahead. Federal support of biomedical research is in danger of being overwhelmed by the national deficit. For the first time in recent years, the President's budget request for the National Institutes of Health did not match the projected rate of inflation and represented an actual decline for many of the individual institutes. Moreover, the Administration seems determined to shift the emphasis from basic toward more applied research.

During the past decade, the present system of peer-reviewed Federal funding, augmented by support from foundations, corporations, and individuals, has played an important role in making American biomedical science the best in the world. Most likely there will now be increased reliance on industrial support at institutions such as ourselves. This will involve important issues concerning intellectual property, conflict-of-interest for scientists and institutions, and just what is an approximate balance between applied and basic research.

Despite such worries, the Laboratory's financial results were very good in 1992. Revenues reached a new high of more than $36 million. For the fourth consecutive year, there was a surplus from operations after adding to reserves for future contingencies and fully providing for depreciation, which in 1992 totaled nearly $2.4 million. The year ended with the Laboratory in strong financial condition.

The past 5 years have been a period of very rapid growth. The number of Ph.D.s here has about doubled, and the academic program now extends from early spring until late fall, with already a few incursions into the winter months. Laboratory scientists have excelled in the competition for Federal and private grant support and so the good financial results are not surprising. Close attention has been directed to controlling operating costs and carefully limiting the growth of support personnel. As a result, the Laboratory has generated a positive cash flow from operations of nearly $10 million before reserves and depreciation over the period. This has been invested in new and improved facilities and programs.

At year-end, the Laboratory's endowment, consisting of the Robertson and Cold Spring Harbor Funds, stood at nearly $65 million, a gain of some $8 million from the previous year. As in the past, the funds have enjoyed excellent investment management by Miller Anderson and Sherrerd and last year also benefited from the addition of over $4 million of new donations to the Cold Spring Harbor Fund. The new money included two new fully endowed chairs of $1.5 million each, which were donated by the W.M. Keck Foundation for the study of structural biology and by the late Maxine Harrison in memory of her mother Alle Davis Harrison for the study of molecular neuroscience. A major factor in the remarkable growth of the endowment has been a conservative drawdown policy, which over the life of the funds has averaged a bit less than 3.5% of income each year. This policy has balanced strong support for science with protecting the funds against inflation and allowing for the support of new programs.

The Laboratory's endowment has been used primarily to fund young scientists who so often produce the most innovative new ideas but have difficulty obtaining early grant support. So it is important that the endowment continue to grow in future years. We cannot realistically expect the recent high rates of in-

vestment return to continue and so adding new funds from new sources must be a high priority.

An important new source of funds is emerging from the Laboratory's technology transfer activities. Federal granting agencies recognize the desirability of technology transfer to the commercial sector for the general public good and therefore encourage grantee institutions to license research results in keeping with the institutions' own policies. As a result, the Laboratory has become more active in commercializing its technology and has received both cash payments and stock ownership in growing amounts as a result of these efforts. The cash and whatever proceeds may be derived from the eventual sale of stock can provide important future support for research. The Laboratory's Board of Trustees has therefore established the "Science Fund" in which to accumulate the proceeds received from such activities. The Science Fund will be an integral part of the endowment intended to be used solely for the long-term support of the direct and indirect costs of the science program.

December 31 of last year marked the formal conclusion of the Second Century Campaign, the Laboratory's enormously successful first ever capital campaign. The total raised of nearly $51 million for facilities, endowment, and programs stands as a remarkable tribute to all involved and as a challenge to those who will be asked to help in future years.

The growing activities of the Cold Spring Harbor Laboratory Press, Banbury Center, and the DNA Learning Center play an important role in the overall success of the Laboratory. They are led impressively by John Inglis, Jan Witkowski, and Dave Micklos, respectively. Each operates as an individual profit center.

Last year, after a difficult 1991, the CSHL Press made a positive financial contribution to the Laboratory in addition to its always positive intellectual contribution. Total revenues exceeded $3.7 million, ahead 20% from 1991. Fifteen new book titles were published, including *The Dynamic Genome*, a remarkable tribute to the late Barbara McClintock, and Jeffrey Miller's eagerly awaited *A Short Course in Bacterial Genetics*.

Revenues of the Journal Department exceeded $1 million for the first time. *Genes & Development,* now in its 5th volume, again earned "most cited" status in its field. It passed the 2600 mark in subscriptions and maintained good profitability. In only its second year, *PCR Methods and Applications* already has over 2000 subscriptions and has been profitable since inception. The success of the journal program is also spawning new journal concepts, one of which, a new journal dealing with the subjects of learning and memory, is already well advanced.

Banbury Center marked its 15th anniversary last year and continues to gain in the esteem of scientists and other professionals in academia, biotechnology, and policy making throughout the world. Sixteen meetings and workshops were held in 1992, covering such diverse subjects as the commercialization of biology, indirect costs, new techniques in molecular biology, human genetics, science policy and funding for the decade of the brain. Of particular significance was a year-end major grant from the Charles A. Dana Foundation for a program to promote research on manic-depressive disease encompassing work here and at Johns Hopkins Medical School and Stanford University Medical School.

The DNA Learning Center (DNALC) had another very productive year, extending its hands-on laboratory workshops on DNA science to increasing numbers of minority and disadvantaged youths. A very successful new program taught genetics to 5th graders at a series of *Fun With DNA* summer camp ses-

sions. The DNALC mobile laboratory vans continued to tour the nation, conducting DNA science workshops for students and teachers.

At year-end, the Laboratory exercised its option to purchase the previously leased DNALC building in Cold Spring Harbor Village from the Cold Spring Harbor School District, and the Learning Center embarked on a two-phase expansion program. Phase I involves a major renovation of the building. It includes the construction of a new 104-seat auditorium with state-of-the-art multimedia audiovisual capability and the installation of a computer teaching laboratory. Phase II will begin a year from now and will include a second DNA science laboratory, a library, and lunchroom facilities.

Of major importance over the past 2 years has been the formation of a Corporate Advisory Committee, now chaired by Doug Fox of *Newsday*. The Committee is composed of representatives of key Long Island companies who are assisting the DNALC in planning a variety of public and professional education programs designed to build support for biotechnology on Long Island and to benefit the economy here. The Committee also seeks to raise badly needed unrestricted funds for the Learning Center from Long Island businesses.

The accomplishments of the past year would not have been possible without the outstanding support of the Laboratory's administrative departments, often operating under difficult financial constraints. Jack Richards and the Buildings and Grounds staff have once again managed a major project, the McClintock Laboratory renovation and expansion, within the budget and on schedule. Art Brings and the Environmental Safety staff have dealt successfully with the seemingly intractable problems of low-level radioactive and medical waste disposal with an innovative program that has sharply reduced the quantity of the materials generated without affecting laboratory efficiency and has saved substantial money. Susan Cooper and her Public Affairs staff have managed an almost endless series of events consistently reflecting distinction on the Laboratory. John Maroney, Bill Keen, Susan Schultz, Sande Chmelev, Cheryl Sinclair, and Barbara Ward also lead their departments with great skill and energy. Roberta Salant assists me immeasurably and tends carefully to the needs of the Board of Trustees.

Now as the calendar turns again we look forward to another of those watershed events that mark the Laboratory's more than 100 years. The Board of Trustees at its February 1993 meeting created the new position of President which Jim Watson will assume as of January 1, 1994. Bruce Stillman, now Assistant Director, will become the Laboratory's new Director at that time. Already Jim and Bruce have been functioning in this way for some time, and it is clear that the leadership of the Laboratory will continue with the vision and strength to which we have all become accustomed.

G. Morgan Browne

BUILDINGS AND GROUNDS

Growth and refinement marked the year of 1992 for the Buildings and Grounds Department as we strove to add more space to our buildings and to refine the facilities of Cold Spring Harbor Laboratory. As 1992 began, the Blackford Hall addition was coming to completion while the McClintock Laboratory renovation stepped up to top speed. Throughout the year, many other projects were started and completed, increasing the Laboratory's usable area and improving its infrastructures.

Blackford Hall

Blackford Hall had already seen 12 months of construction as 1992 began and the deadline for the spring meetings was fast approaching. Renovation of the hot kitchen, the final phase, began in January. This work included the installation of new appliances in their new location, completion of new cold storage rooms, removal of the old hot kitchen, and installation of the new cold kitchen and serving lines. At the same time, finish work was being completed in all other areas of Blackford Hall; all surfaces received a final coat of paint, carpet was put down, new furniture was put in place, and the area surrounding the building was landscaped. Because the project posed significant challenges, it was decided to use as much laboratory labor as possible to facilitate timing and coordination. For example, during the entire renovation, the construction crews did their best to work in areas that would be least disruptive to the food service staff while they continued to prepare and serve many meals. Our electricians, plumbers, carpenters, painters, and HVAC (Heating, Ventilating, and Air Conditioning) staff all worked extremely hard to meet the construction deadline and produce a top quality building. To the delight of all, the work at Blackford Hall was completed and the spring meetings season of 1992 had a new and expanded dining hall.

McClintock Laboratory

1992 found the McClintock Laboratory building very empty and in the initial stages of demolition. To provide a first-hand look at the physical construction of this very old building, some of the walls and ceilings were removed so that the architects and engineers could proceed with their design work. Once all the design problems were solved, demolition proceeded rapidly and the entire building was gutted. Slowly, some of the walls were put in, and the elevator pit and shaft were installed. Once again, Blackford lawn was dug up so that a the new water line could be run to McClintock. As the year came to a close, McClintock Laboratory had a new third floor, and much of the mechanical equipment and new windows had been installed. The building is well on its way to being completed in the spring of 1993.

Alterations and Renovations

Jones Laboratory: Jones had always been used as a summer course laboratory, with limited winter use. However, because of increased space requirements for both laboratories and courses, Jones Laboratory was converted into a full-

fledged lab by the Buildings and Grounds staff in only 4 months. Included in this project was the relocation of the cold room, complete overhaul of the HVAC systems, installation of vacuum and compressed air, the addition of a glassware kitchen, installation of office space, complete repainting, and extensive work on the electrical systems.

Bassett House: Located on the Uplands Farms facility, Bassett House was transformed from a dorm type of residence to a single-family dwelling for one of our scientists. The entire interior was repainted, and all of the floors were refinished or carpeted.

Doubleday House: Renovation of this house on Moore's Hill Road included construction of a new deck to replace the old one. In addition, the exterior of the house was painted and the surrounding areas were relandscaped. Doubleday House will also provide quality living space for Laboratory staff.

Energy Projects

Two Buildings and Grounds projects that have had significant impact on our facilities are the replacement of the James emergency generator and the ongoing installation of Building Energy Management. It was decided in the spring of 1992 that the James generator had come to the end of its usefulness. The unit needed extensive repairs and the building was demanding more power than the generator could produce. A larger unit was thus purchased and installed in a new location. All of the trades contributed to the installation of the new unit, including the electrical crews who spent long hard hours rewiring the emergency circuits. All of our crews are to be congratulated for a fine job.

The HVAC Department has undertaken a long-term modernization project that is starting to show favorable impact on the management of our energy consumption. During the year, the old controls on many of the mechanical systems were replaced with computer-driven controls. This replacement included complete rewiring of the HVAC systems, installation of new controls, and programming for maximum efficiency. These technological enhancements have made it easier to monitor and correct problems before they become major difficulties. As the year closed, the HVAC crew completed work on Grace Auditorium, Bush Lecture Hall, Blackford Hall, Jones Laboratory, Beckman, and much of Demerec Laboratory. Many years of work will be required to complete this project, but the benefits of lower energy costs and less mechanical wear are already very significant.

Core Facilities

Some of the core facilities under the Buildings and Grounds Department will see some changes in 1993. We started in 1992 with the purchase of a golf cart for the Equipment Repair Department to help with the many trips up and down to the Beckman Building. Bob Borruso of this department retired after 18 years of service to the Laboratory. We wish him well in his retirement. We transferred George Newell from the Grounds Department to the open position created by Bob's retirement, and we added a new position, Equipment Maintenance Technician, filled by Danielle Strano. With two new people to train and the increasing work load, Cliff Sutkevich will have his hands full.

Next on the list for an overhaul is the Receiving Department, whose work load has become overwhelming. We will look for ways to improve the operation of this department in 1993.

Thanks to All

The Buildings and Grounds Department has completed another year. The talented people in all our departments have worked very hard to keep up with the Laboratory's increasing work load, and I want to thank them all for a job well done. Special thanks also go to the office staff; Pege, Charlie, Leslie, and Peter. Finally, Peter Stahl is doing a great job as our new Facilities Manager; he is a most valuable employee and a great help to me.

Jack Richards

DEVELOPMENT

1992 and Beyond

1992 was another very successful year for development at the Laboratory. The highly successful Second Century Campaign raised $5,139,689 in 1992 and totaled $51,092,883 at the end of the year (with prospects of more to come in 1993 from solicitations currently outstanding). This amount included, in part, gifts of $2,034,418 to the Endowment Fund (which stood at $32,396,431 on December 31), $1,500,000 to the Cancer Research Fund, $650,000 to the Infrastructure Fund, and $350,000 to the Education Fund.

The Annual Fund of the Cold Spring Harbor Laboratory Association (CSHLA) contributed $531,263 in 1992, exceeding $500,000 for the first time. Even without an organized effort, the laboratory received Planned Gifts totaling $567,278.

This is a very healthy growth pattern not only because of the actual amount of the contributions received, but also because of the diversity of giving sources. The Laboratory's continuing reputation for excellence in the field of basic research in genetics attracts important support from Trustees, Directors of the CSHLA, other individuals, foundations, and corporations; this breadth of support from a variety of sources outside the federal government is very desirable.

As we look to 1993, the Laboratory's needs will be concentrated in four areas: increasing the Endowment, organizing a comprehensive Planned Giving Program, building the Annual Fund, and, on a rifle shot basis, raising funds for specific capital projects. As our operating budget grows and Federal funding becomes ever more fickle, we want to strenghten our financial foundation by making a concerted effort to increase the Endowment and develop an effective

Planned Giving program; at the same time, and with equal enthusiasm, we want to increase the level of Annual Giving in order to provide additional funding for our science program. Finally, we will probably need to raise some capital funds from time to time, but only for specific projects and at a lower level of intensity than in the past.

The Laboratory is fortunate to have so many good friends and loyal supporters. We are most appreciative of the breadth and depth of this support and thank each of you for what you have done for the Laboratory over the years; we hope you will continue to be firm in your support as we embark on these new directions in development.

Second Century Campaign

As part of the Laboratory's Centennial celebration in 1990, a major fund-raising campaign—the Second Century Campaign—was organized with a goal of raising $44 million in support of capital construction projects and the establishment of endowment funds to support the programs of the Laboratory. At the end of 1992, gifts and pledges totaling $51,092,883 had been raised, exceeding the goal by $7,092,883. In addition, we think there will be additional contributions coming in during 1993, in response to currently outstanding solicitations.

This has certainly been a tremendously successful effort, with broad support from individuals, foundations, corporations, and Trustees. However, it is fair to say that the Trustees contributed more than their fair share to this effort not only with their gifts and pledges, but also with their leadership. David L. Luke III, now Chairman of the Board of Trustees, was Chairman of the Second Century Campaign. He was blessed with an outstanding Steering Committee consisting of George W. Cutting, Jr., Oliver R. Grace, Mrs. Sinclair Hatch, Townsend J. Knight, William R. Miller, and Taggart Whipple.

During 1992, the Second Century Campaign received gifts and pledges totaling $5,139,689 from a broad base of donors. The Trustees again showed their strong support for the work being done at the Laboratory, with Bill and Ellie Everdell, George and Mary Lindsay, Edwin and Nancy Marks, and Tom and Jordan Saunders making wonderfully generous gifts to the Cancer Research Fund.

The Laboratory enjoys an excellent reputation with foundations, and they continue to provide very substantial support for our activities. We received a spectacular gift for our Endowment from the W.M. Keck Foundation, which enabled us to establish a Chair in Structural Biology; the William Stamps Farish Fund made a most generous grant to the Cancer Research Fund, as did the Bodman and Achelis Foundations and the Hyde and Watson Foundation. Our Endowment received another wonderful boost from the Ft. Hill Foundation which made it possible to start the William T. Matheson Chair. The Robertson family, with Bill Robertson serving as a Trustee of the Laboratory and Anne Meier as a Director of the Association, demonstrated their generosity again in 1992 with an additional contribution to the Charles Robertson Chair in Neuroscience; Bob Gardner also made an additional contribution to the Second Century Campaign, as did the William and Maude Pritchard Charitable Trust. Early in the year, we received a very significant bequest from the estate of Charles Henry Leach II.

Corporations again gave wonderful support to the Laboratory in 1992 with grants received from the Baxter Foundation, Marion Merrell Dow Inc., J.P.

Morgan and Co., Incorporated, and Pfizer Inc. and an additional contribution from Westvaco Corporation.

Mary Jeanne and Henry Harris are two individuals who have done much for Cold Spring Harbor Laboratory over the years, with particular emphasis on the DNA Learning Center. In 1992, they made another generous contribution to the Education Fund, which supports the programs of the DNA Learning Center. Cablevision also made a very generous contribution to the Education Fund.

Our heartfelt thanks for all of this support and for the very effective effort of all the volunteers. The Laboratory is certainly the richer for the success of the Second Century Campaign not only because of the additional laboratories, visitor facilities, and increased endowment, but also because of the deep involvement of the Trustees, the Directors of the CSHLA, and the broad base of wonderful volunteers, all of whom worked so hard on this campaign.

Annual Giving

Annual Giving at Cold Spring Harbor Laboratory really consists of four parts: the CSHL Association Annual Fund, the Corporate Sponsor Program, the Undergraduate Research Program, and the DNA Learning Center Annual Fund. These four entities contributed a total of $1,443,440 to the Laboratory in 1992, a handsome sum in and of itself and a significant increase over 1991.

The CSHLA Annual Fund, the major source of unrestricted giving to the Laboratory, contributed a total of $531,263 in 1992, of which $436,540 was in unrestricted funds. This was the first time the Annual Fund exceeded the $500,000 level, an important milestone for the Laboratory and a particular tribute to the efforts of the CSHLA and George Cutting, who has led the Association since 1986. The Annual Fund, which is devoted to science, was up by almost $100,000 in 1992 compared to 1991, a tremendous vote of confidence in the work of the Laboratory and of great importance to the continuation of the important work done here.

The Corporate Sponsors Program, which supports the meetings program at the Laboratory and at the Banbury Center, generated $741,000 from 38 companies in 1992. This represented an increase from 1991 not only in total support, but also in number of companies participating.

Undergraduate Research Program, a highly selective 10-week summer program for college students interested in basic scientific research, is supported from a wide variety of sources and raised $128,404 in 1992. Each student—the group usually numbers about 20 and is selected from applications from around the world—is assigned a research project by a staff scientist, who becomes that student's mentor. This is an extraordinary opportunity for the students selected to work with a top scientist here at the Laboratory.

The DNA Learning Center Annual Fund, an initiative begun in 1991, is designed to create a base of unrestricted annual support for the DNA Learning Center from the Long Island business community. This effort, which is organized by the Corporate Advisory Board, was originally headed by Robert McMillan of McMillan, Rather, Bennett & Rigano, P.C. and his efforts got the DNA Learning

Center Annual Fund off to an excellent start. Douglas B. Fox, Vice President of Marketing, Times Mirror Group, took over the chairmanship early in 1992 and devoted considerable effort working on a mission statement to make the DNA Learning Center more meaningful to the business community. The DNA Learning Center Annual Fund generated $29,100 in 1992 and is positioned to grow significantly in 1993.

Planned Giving

Planned Gifts are of great benefit to the Laboratory. This method of giving frequently enables donors to make a larger gift than they had thought possible and, at the same time, can provide them with significant economic and tax advantages. These can be benefits of increased income, elimination of capital gain taxes, and/or current tax deductions. Planned Gifts typically go to the Endowment which, as the Laboratory grows in size and scope, becomes an ever more important part of our financial foundation. Planned Gifts can be made to take effect immediately or be deferred to take effect at a later date. Planned Gifts fall into three basic categories:

Bequests: Perhaps the easiest and best known method of planned giving in which donors make a provision in their will for Cold Spring Harbor Laboratory.

Life Income Plans: Perhaps the most popular planned gifts are the various life income plans that benefit both the donor and CSHL during the donor's lifetime. In a typical life income plan, the donor transfers cash, securities, or other marketable assets in return for a life income for self, spouse, and/or others, with the remainder principal passing to the Laboratory at the death of the last surviving beneficiary. The donor gains a current income tax deduction for the present value of the projected remainder interest. If appreciated assets are used to fund the life income plan, the donor avoids capital gains tax on the transfer.

There are three basic kinds of Life Income Plans: The CSHL Pooled Income Fund (managed by U.S. Trust Company); a Gift Annuity or Deferred Gift Annuity; and a Charitable Remainder Trust.

Other Planned Gifts: These include Charitable Lead Trusts, Life Insurance, and Retained Life Estate.

If you are interested in discussing a Planned Gift to the Cold Spring Harbor Laboratory, please call Gordon S. Hargraves in the Development Office (phone: (516) 367-8842). We can help you get started.

Staff

The staff of the Development Department is quite small in relation to the amount of money raised for the Laboratory, but our efforts are leveraged enormously by the number and excellence of the volunteers who contribute their time and expertise to make things happen.

The Development Committee of the Board of Trustees helps to keep our efforts and priorities focused, so that staff resources are well utilized. The Directors of the CSHLA assist us by shouldering many burdens in helping to fulfill the mission of the Association.

We are lucky to have such a hard-working and effective staff in the Development Department, and the staff, in turn, is fortunate in having such an extraordinary cadre of volunteers with which to work. Particular thanks go to Joan Pesek, now Associate Director of Annual Giving; Claire Fairman, Events Coordinator; and Debra Mullen and Gisela Jennings, secretaries, all of whom churn out an enormous amount of good work with a high degree of cheerfulness.

Gordon S. Hargraves

LIBRARY SERVICES

Staff Changes

Genemary Falvey, head of library services, resigned in July to relocate to Anchorage, Alaska, where her husband, John, will be teaching at the university there. Genemary had been with the library for more than 10 years, first as a volunteer, then as trainee and acting librarian. After completing her MLS in 1981 at Long Island University, C.W. Post campus, Genemary became the librarian. During her tenure at the Laboratory, she began the task of computerizing the library's technical and reference services. Working closely with the scientists and computer center, Genemary installed several reference databases, including the CD-ROM which uses compact disks to impart information to users. These disks hold huge amounts of data and have excellent graphic resolution. By using a tower that holds several CDs, information can be accessed concurrently. Beginning February 1993, Genemary will become science/medicine librarian for the Anchorage Municipal Libraries. She will be headquartered in the Z.J. Loussac Central Library.

Margaret Henderson, the new head of library services, came to us from the DNA Learning Center where she taught science classes. Prior to coming to the Laboratory, Margaret was the reference/on-line services librarian at the University of Western Ontario, London, where she received her MLIS in 1986. Mrs. Henderson will continue to bring the library's catalogs on line and to streamline public services.

Lynn Kasso, archives assistant, left in June and relocated to Arizona. Lynn was largely responsible for the move from the archives room to an archives floor in 1988. She provided accurate, secure access to this repository of Laboratory history. Lynn cataloged the archival holdings, putting many of the manual records into the computer.

Our new archives assistant, Clare Bunce, was promoted from the library clerk position, where her experience in the library and public affairs made her an excellent candidate. Replacing Clare is Kelly Kasso, a recent graduate of SUNY College at Oswego. Kelly's education and her interest and energy have already made her a valuable asset to this small hard-working staff.

Continued Active Archives

1992 was a busy year for the archives. With the death in September of Barbara McClintock, requests for information about her life increased twofold. Dr. McClintock touched the lives of so many people; requests have come from the press, students, teachers, scientists, and artists, all with a common interest in her life.

There has also been heightened interest in Dr. Watson and his relationship to the Human Genome Project as well as the approaching 40th anniversary of the discovery of the structure of the DNA double helix. Requests for reprints, biographical information, and photographs have increased.

The subject of the 1992 Archives Conference held at Brookhaven National Laboratory in October was "Discovery." We exhibited important reprints of books by Alfred Hershey, James D. Watson, and Barbara McClintock, as well as books detailing the Laboratory's history. A montage of photographs of Dr. McClintock from her youth through her last days here was displayed, surrounded by corn and her published works. This exhibit drew interest from all who attended.

Reference Services

An additional computer has been added for bibliographic searching. Having two computers in the reference room allows more patrons to access Medline, Entrez-Sequences, and Current Contents. These computers also provide access to the Laboratory network and word processing and also have the full text of the *Journal of Biological Chemistry* available. We will continue to acquire additional bibliographic and text CDs as the budget permits.

The library staff answered more than 200 reference questions per month in 1992. The tables of contents of 87 journals continue to be sent to 60 scientists. In September, Margaret Henderson began the *Library Newsletter*, whose purpose is to inform the staff of new books, new journals, and new and changing library policies.

The on-line serials system has been installed and includes the use of Internet to claim missing and damaged issues of journals. This provides needed assistance to Leigh Johnson whose considerable talent as library assistant can be utilized in other areas. More than 5000 books have already been entered into the on-line database and will be available to the scientists in 1993. The catalog will be accessible directly from scientific offices, laboratories, and branch libraries.

Whatever the medium—whether it be paper, compact disk, electronic, audio, or video cassette—the mission of the library continues to support the information needs of the scientific staff, quickly and accurately.

Space and Water Damage

Since the need for additional space has not yet been addressed, we must continue sending older journals to off-premises storage in order to provide space for the current journal collection. The need for study space has become extremely critical. Quiet study areas are in short supply.

Water damage plagues the lower level, especially the Watson archives, where puddles contribute to an alarming increase in mold and staining. The time has come to address the future plans for the Laboratory's information center. It is no longer acceptable to ignore and postpone decisions that affect the library's future. I urge the administration to address this issue immediately.

Susan Cooper

PUBLIC AFFAIRS

Events

A sizable number of events were planned and organized this year by the Public Affairs Department. On January 25,1992, concert pianist Peter Orth performed a second benefit for Cold Spring Harbor Laboratory. The capacity audience was thrilled with Orth's performance of Chopin, Schumann, and Brahms. The net income from this benefit was $8000.

The official end of the Second Century Campaign was discussed on April 12,1992, when Chairman David Luke III announced that the campaign had successfully raised $6 million over the goal of $44 million. The dinner held in the new dining room in Blackford Hall was a celebration of the committee's achievement and the announcement of the fact that the deadline was being extended until December 31,1992, with the exception of solicitations outstanding.

The Samuel Freeman Cancer Center in the newly renovated Jones Laboratory was dedicated on July 19, 1992. Dr. Robert Franza spoke about the new research that is being done in the Center and remarked on the joys of being in a building with such historical importance. Elizabeth Watson, *Architectural Historian,* presented a brief history of the Jones building, and Dr. Watson's talk centered on the recognition of the Samuel Freeman Charitable Trust and its Chairman, William E. Murray. Dr. Watson praised Mr. Murray for his continuous support of special Laboratory projects and thanked him on behalf of the Board of Trustees. The afternoon concluded with a dinner at Airslie.

A special dinner was held on June 6,1992, to recognize George Cahill's assistance with the Neuroscience Center during his tenure at the Howard Hughes Medical Institute. Dr. Cahill, his wife Sally, members of his family, and personal friends had dinner with Dr. and Mrs. Watson at Airslie house.

Barbara McClintock delighted in her 90th birthday party held at Airslie house. Thirty close friends from all phases of Dr. McClintock's life came to Cold Spring Harbor on June 13 to share stories and laughter and to unveil *The Dynamic Genome*, a book with contributions from many more of her friends and colleagues and produced by Cold Spring Harbor Laboratory Press. She told us that she was surprised to have enjoyed the celebration. "The food was good; I had some wine and probably talked too much. It was fun, but I'm very tired," she said.

Sadly, Dr. McClintock died on September 2, and on November 17 a celebration of her life was held in Grace Auditorium. Many of her friends gathered to hear Howard Green (Harvard University), James Shapiro (University of Chicago), Evelyn Witkin (Rutgers University), and Oliver Nelson (University of Wisconsin, Madison) speak on the many aspects of Barbara's life. A small program was prepared for the occasion with an elegant essay by Nathaniel Comfort entitled "Small in size, but great in stature." Waclaw Szabalski (University of Wisconsin, Madison), Jochim Messing (Rutgers University), and Tom Broker (University of Rochester) made special comments at the end. A collection of these talks is being prepared for distribution in late spring 1993.

On October 4,1992, the Racker Reading Room in Blackford Hall was dedicated. Mrs. Franzie Racker presented the Laboratory with 22 of her husband's drawings. In the 1940s and 1950s, Dr. Efraim Racker sketched the scientists who worked and studied at Cold Spring Harbor Laboratory. Their likenesses now adorn the walls of Blackford Hall. Scientists from that period were invited back to reminisce, and so they did: Rollin Hotchkiss, Ernst Mayr, Bruce Wallace, and Jim Watson imparted interesting tales of times past. The decoration of this room, made easier by its rich history, would not have been possible without the aesthetic and artistic choices made by Laura Hyman and Liz Watson.

In addition to these major events, four Lloyd Harbor Seminars were planned and presented with the help of George Toumanoff, a Lloyd Harbor Trustee. These seminars are held at the Banbury Center in February, March, October, and November. The 1992 seminars were The Living Will and New York's Proxy Law; Science in Russia after Glasnost; Myths and Realities of the Soviet Union; and Understanding Learning and Memory.

All four regularly scheduled public walking tours were given to a total of 106 new faces. Special talks and tours were arranged for Svalovskommun, Svalov, Sweden; C.W. Post University Club, Greenvale; The Japanese Exchange Team, Syosset Rotary; Alternative Culture Exchange, Jericho; C.W. Post Special Library class, Lawrence Road Jr. High School; Northport Girl Scouts, Commack Middle School; Aspect ILS (international students); Ethical Humanists Society of Long Island; Cedar Swamp Historical Society; C.W. Post Library Association; Polytechnic University Alumni Association; Independent School Exchange, Greenvale; and Kiwanis Club of Huntington. I am grateful to the many laboratory scientists and other staff members who made these tours and talks a rich resource for our community.

Several holiday parties were organized by the staff Activities Committee co-chaired by Art Brings and David Helfman. The new tree-lighting and caroling event on December 9 was a special success and will continue annually. Santa Claus distributed presents to 102 Laboratory kids at the first Lab-wide children's holiday party held on December 16. The gifts were purchased by Joan Pesek, Lesley Inglis, Candy Davis, and Grace Stillman and wrapped by a dozen volunteers from the staff. The party included holiday goodies and well-deserved wine and cheese for the parents in attendance. Plans for other parties included Super Bowl Sunday, Valentine's Day, St. Patrick's Day, Halloween, and Crazy Hat Day, all arranged by Rob Gensel. The annual Thanksgiving Day feast, organized with good help from Margaret Henderson, returned in 1992 after a 3-year hiatus while Blackford Hall was being renovated.

As a member of the newly convened Events and Benefits committee under the leadership of board vice-chairman Mary Lindsay, I now include among my responsibilites the generation of a monthly Laboratory-wide calendar. To this

end, I installed Calendar Creator Plus software on my personal computer to produce the calendar. This speeds up the process of informing staff and volunteers of the wide variety of changing activities at the Laboratory.

New Printed Materials Produced by Veteran Staff

Harbor Transcript has a new look and new colors. Margot Bennett, using her new Mac IIci and Quark Express design software, fashioned a new masthead and format beginning with volume 10, and our first century burgundy has given way to a second century blue. The 12-page design has some standard features: The front page stories always share the limelight equally between scientific research and non-science Laboratory-related information; alternate issues trade trustee profiles with historical highlights; and a new feature, the book review, appears in each issue on page 11. This design lends itself to easier preplanning while retaining the flexibility necessary to meet the changing information demands of this institution. Margot's artistic talent and special nurturing of the *Harbor Transcript* as well as all the other printed pieces create a visual image that equals the quality of the institution.

Nathaniel Comfort has brought new substance to the writing of the *Harbor Transcript* and the other pieces produced for the Laboratory. He has the tenacity and ability to capture the essence of a scientific project and interpret that to our audience. Nathan's research skills and the sheer volume of work he produces are overwhelming. One of his major contributions to the press package this year was the conceptualization and creation of the *Experts Book*. This small volume details and indexes the work of individual researchers and encourages the press to seek answers to questions in molecular biology from our Laboratory scientists. The cover is a terrific cartoon drawn by CSHL artist Jim Duffy.

In addition to the 1992 issues of the *Transcript*, Public Affairs produced three meetings and course posters; *Take a Look at Us*, a handout for the general public, and the *Annual Fund* brochure with its accompanying solicitation cards and envelopes. Two pieces that accompany our public handout, the Trustee Card and the Fact Sheet, were each updated twice in 1992. Four event invitations, three programs, and five fliers were produced and widely distributed. *F.A.C.E.S.*, the Laboratory photographic yearbook, will skip a season and be issued in April, 1993, and annually thereafter.

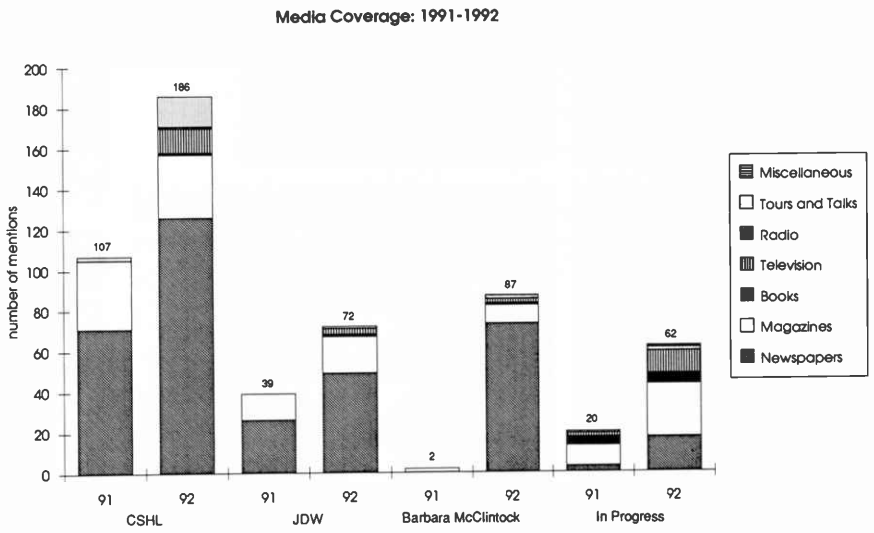
Events Challenge Audiovisual Department

In 1992, the Laboratory held more than 30 scientific meetings either in Grace Auditorium or at Banbury, more than 15 Association and public events, three concerts, and two dedications, as well as in-house events too numerous to count. Herb Parsons and Ed Campodonico, with the help of some excellent part-time support in the summer, manage to handle the audiovisual (AV) needs of this complex organization. They juggle individual course-site requests for VCRs, projectors, and microphones and provide light and sound for special events from the end of summer party, through the small group of folk dancers who meet on Mondays, to the needs of a world-class concert pianist. The ever-increasing number of events has stretched the AV staff and facilities to the farthest point.

We are no longer assured that Banbury meetings will not run concurrently with an event planned for Grace Auditorium or that one of our AV specialists will

be able to double as a photographer. The time has come to reorganize the department and add the staff necessary to support this vital function.

Media Coverage Increases

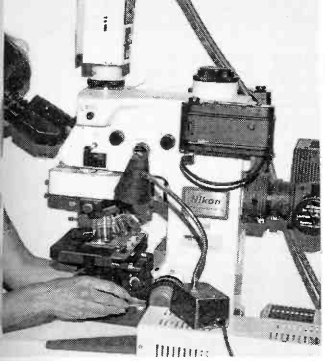
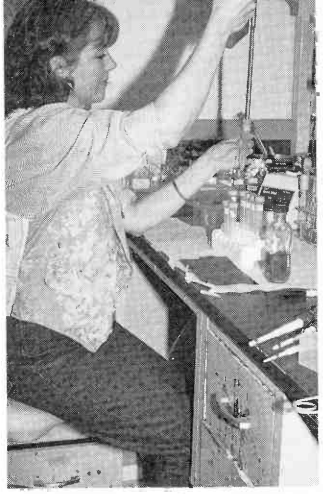
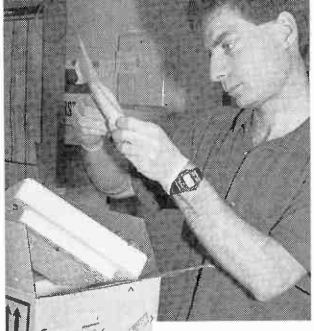


Comparison of media coverage for 1991 and 1992. CSHL= coverage of the Laboratory; JDW= coverage of James Watson and the Human Genome Project; In Progress= coverage in any of the three preceding categories unpublished as of the end of the year.

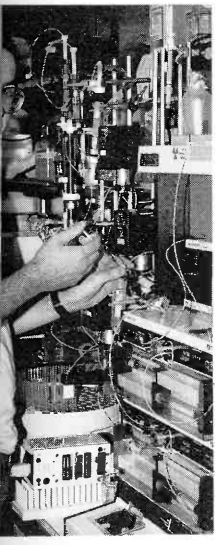
As the chart implies, Public Affairs was flooded by requests for information about the Laboratory and its staff. A memorable press year saw telomeres and origin recognition complex (ORC) become the focus of the scientific press. The resignation of Jim Watson as Director of the National Center for Human Genome Research and the death of Barbara McClintock created intense peaks in the Department's media work, yet there was still more general coverage of the Laboratory than in any previous year. Both of these peak events were reminders of our vulnerability as well as indications of individual strength. Jim Watson's stellar 4-year government career brought worldwide attention and support for the Human Genome Project, which now reveals daily some clue to our genetic makeup. Barbara McClintock, friend and colleague for 20 years, Laboratory employee for more than 50 years, and a lover of life for more than 90 years will be missed personally, professionally, and scientifically. Her legacy of elegant observation, keen wit, and scientific clarity will guide many for centuries to come.

With the events calendar already well into 1995, it is clear that this Department will not want for work. Plans already include the dedication of Blackford Hall and McClintock Laboratory and the completion of a new four-color booklet detailing our present scientific program.

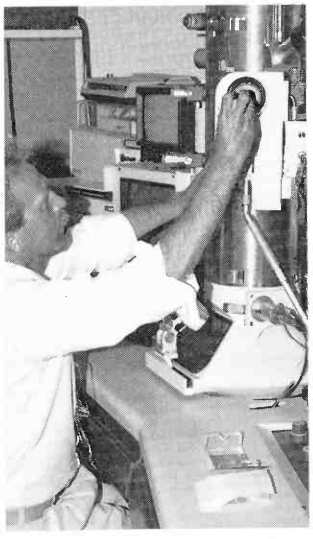
Susan Cooper

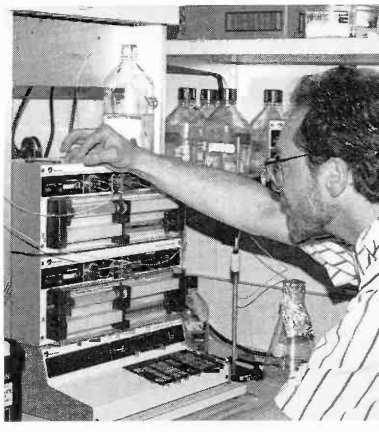





RESEARCH









Row 1: Shelby Landon, Patricia Elliott, Bruce Futcher, Frank Pessler
Row 2: Adrian Krainer, Lea Harrington, Celeste Casciato, Cynthia Sadowski
Row 3: Cecelia Devlin, Robert Derby, George Tokiwa, Thomas Melendy
Row 4: Hai Huang, student, Robert Nash, Catherine Flanagan

TUMOR VIRUSES

Members of the Tumor Virus Section are wedded by an interest in understanding cellular processes in eukaryotes and how they are altered in cancer cells. When this section began nearly 25 years ago, tumor viruses were the tool used to probe these mechanisms. Nowadays, the number of tools available is greatly expanded. In addition to the DNA tumor viruses, SV40, adenovirus, and papillomavirus, section members use human immunodeficiency virus and herpes simplex virus as probes. In recent years, we have learned that eukaryotes as outwardly different as yeast and humans are more similar than dissimilar. Yeast is therefore becoming an increasingly popular model to study cell processes relevant to human cells. With these various tools, members of this section study (1) the structure and replication of viral and cellular chromosomes (Stendlund, Stillman) and regulation of this process through the cell cycle (Mathews, Moran, Morris, Stillman); (2) regulation of transcription (Herr, Laspia, Mathews, Morris, Tanaka) and the processing of the resulting transcripts (Krainer); (3) translational control (Mathews); and (4) regulation of these processes by phosphorylation (Marshak). The studies by these investigators are described in this section.

DNA SYNTHESIS

B. Stillman N. Muzyczka, M. Akiyama, G. Bauer, S.P. Bell, S. Brill, S.-U. Din, A. Dutta, C. Hardy, P. Kaufman, T. Melendy, J.M. Ruppert, S. Waga, F. Bunz, K. Fien, K. Gavin, Y. Marahrens, H. Rao, L. Borzillo, N. Kessler

DNA is the genetic material present in all cells and needs to be accurately duplicated with each cell division. The enzymatic process of replicating the double helix is conserved from bacteria to human cells. In particular, the general process of initiation of replication is much the same for the bacterial chromosome of *Escherichia coli*, the bacteriophage λ chromosome, and the chromosome of the small DNA virus, SV40. Although we know that eukaryotic cells have specific DNA sequences that specify the initiation of DNA replication, the mechanism and control of this process are not understood. It is commonly expected that initiation of DNA replication within eukaryotic chromosome replicons will follow the paradigm established for other well-studied replicons, but we simply do not know how eukaryotic chromosomes initiate DNA replication. Our recent work has increasingly focused on this latter problem.

SV40 DNA Replication

F. Bunz, L. Borzillo, S.-U. Din, A. Dutta, T. Melendy
N. Muzyczka, J.M. Ruppert, S. Waga, B. Stillman

As an entrée into the study of DNA replication in eukaryotic cells, we have for many years studied the replication of the small chromosome of simian virus 40 (SV40). This is because the SV40 chromosome has a single replicator that determines the site of initiation of DNA replication, called the SV40 origin (*ori*) sequence. Moreover, only one virus-encoded protein, the SV40 large (T) tumor antigen, is required for the replication of the small chromosome. Thus, DNA replication relies heavily on the cellular replication apparatus. For some years, we have purified proteins from human cell extracts that are required, with T antigen, for the replication of SV40 *ori-*

containing plasmids. We previously have reconstituted with purified proteins the initiation of DNA replication, the replication of both leading and lagging strands, and the segregation of the daughter chromosomes. In this purified system, however, the lagging-strand Okazaki fragments remained as short, discontinuous strands because they were not ligated together. We have therefore focused our attention on reconstituting the complete replication of SV40 *ori*-containing plasmids and the production of a covalently closed, circular product in which the lagging-strand Okazaki fragments become ligated during DNA replication.

Two new DNA replication factors, maturation factor 1 (MF1), a 5′ to 3′ exonuclease, and DNA ligase 1, have been identified as essential components required for the complete reconstitution of SV40 DNA replication. These two proteins are required along with the previously identified DNA replication factors summarized in Table 1, and all of these purified DNA replication factors are necessary and sufficient in the reconstituted replication reaction. It has therefore been possible to determine the role of each factor in complete DNA replication by systematically omitting single factors from the reconstituted reaction and examining the corresponding "phenotype" by determining the structure of the resulting replication products. Utilizing this approach, we have demonstrated that RPA, polymerase α/primase, and T antigen are essential for the initiation of DNA replication. Separate omission of RFC, PCNA, and DNA polymerase δ eliminated leading-strand synthesis, as we have demonstrated before, but allowed the synthesis of Okazaki fragments on the lagging-strand template. Surprisingly, in the presence of MF1 and DNA ligase, these Okazaki fragments were not ligated together, suggesting that PCNA, RFC, and DNA polymerase δ are required for complete synthesis of the lagging-strand product. We are currently investigating how these three DNA replication components are required for lagging-strand synthesis, but the simplest hypothesis is that they are required to extend short nascent DNA chains that are synthesized by DNA polymerase α/primase so that DNA ligase and MF1 can cooperate to ligate the nascent Okazaki fragment to the growing lagging strand. This and other possible explanations are under further investigation.

TABLE 1 DNA Replication Factors

Protein	Functions
T antigen	initiator; DNA helicase; primosome loading protein
Replication protein A (RPA)	single-strand-binding protein; polymerase accessory protein; initiation cofactor
DNA polymerase α/primase	DNA polymerase and DNA primase for lagging strand
DNA polymerase δ	DNA polymerase for leading and lagging strand
Replication factor C (RFC)	primer-template-binding protein; ATPase; polymerase accessory factor
Proliferating cell nuclear antigen (PCNA)	polymerase δ accessory factor; RFC cofactor
Topoisiomerase I	removes tortional strain;
Topoisiomerase II	chromosome segregation
Maturation factor 1 (MF1)	5′ to 3′ exonuclease required for lagging-strand synthesis
DNA ligase I	DNA ligase required for lagging-strand synthesis

As a part of our effort to understand the mechanism and regulation of cellular DNA replication, we have been systematically cloning cDNAs encoding the human DNA replication proteins. In collaboration with Dr. Ryuji Kobayashi (see Protein Chemistry, this section), protein sequence has been obtained from the five subunits of the human RFC protein, and a full-length cDNA encoding the 140-kD large subunit of RFC has been obtained. In addition, a panel of monoclonal antibodies raised against the 140-kD RFC subunit has been prepared, and these reagents are currently in use to determine the precise functions of the polypeptide.

As indicated above, T antigen, RPA, and DNA polymerase α/primase are essential for the initation of DNA replication, and in their absence, no nucleic acid synthesis occurs. In the last year, results from a number of laboratories have established that specific protein-protein interactions between these replication proteins play a critical role in establishing a functional initiation complex at the SV40 origin of DNA replication. T antigen interacts with DNA polymerase α/primase (composed of four polypeptides) and with RPA (composed of three polypeptides). Moreover, both RPA and DNA polymerase α/primase interact with each other.

We have investigated the functional consequences of these protein-protein interactions by testing the ability of DNA polymerase α/primase to initiate DNA replication on unprimed, single-stranded DNA templates. When the single-stranded DNA is coated

with RPA, DNA polymerase α/primase is unable to initiate DNA synthesis. Addition of T antigen, however, allows DNA polymerase α/DNA primase to initiate DNA replication on this template DNA. On the basis of our results and the results from other laboratories, we have proposed that T antigen, a DNA helicase and initiator protein, also functions to load DNA polymerase α/primase on to the RPA-coated template DNA much like the primosome loading proteins found in prokaryotes. For example, the *E. coli* dnaC protein is required to load the dnaB helicase on to single-stranded DNA-binding protein coated DNA, thereby allowing *E. coli* primase to bind to the template DNA and prime DNA synthesis. We imagine that T antigen functions like dnaC as a primosome loading protein. Therefore, T antigen has combined the function of three separate *E. coli* replication proteins: the initiator function (dnaA), a DNA helicase function (dnaB), and a primosome loading function (dnaC).

Because initiation of replication from the SV40 *ori* relies so heavily on T antigen, it is not possible to extrapolate these studies on SV40 DNA replication to reveal details about the initiation of the replication of cellular chromosomes. Therefore, we have increasingly turned our attention to studying the replication of chromosomes in the yeast *Saccharomyces cerevisiae*. Furthermore, to address more directly the role in chromosome duplication of the cellular replication factors described in Table 1, we have systematically purified the functional homologs of these human cell proteins from *S. cerevisiae* and have cloned the genes encoding them so that a genetic approach may yield insight into their function.

Cell Chromosome Replication

M. Akiyama, S.P. Bell, S. Brill, K. Fien, K. Gavin
C. Hardy, Y. Marahrens, H. Rao, B. Stillman

As discussed above, we have begun to identify from *S. cerevisiae* the functional homologs of the human DNA replication factors that are required for SV40 DNA replication in vitro. To date, all of the replication factors (except MF1) have been identified, and many of the genes encoding subunits of these proteins have been cloned by us or others. Most recently, RFC from *S. cerevisiae* (scRFC) has been purified, and in collaboration with Dr. Ryuji Kobayashi, protein sequence has been obtained to clone the genes encoding each subunit. These studies, and similar studies with scRPA reported previously, will allow a genetic approach toward an understanding of the roles of these proteins in cellular DNA metabolism.

One of our central goals is to understand how cellular DNA replication begins and how this process is controlled. To this end, we have previously studied the structure and DNA sequence requirements for a chromosomal replicator or origin of DNA replication called *ARS1*, located on chromosome IV in *S. cerevisiae*. Linker scan mutagenesis of the locus revealed that *ARS1* contains one essential (A) and three important (B1, B2, B3) DNA sequence elements. The individual B elements are not essential, but combined, the B elements are necessary for *ARS* function. The B3 element binds the cellular transcription factor ABF1 and the A element is responsible for sequence-specific recognition by the origin recognition complex (ORC), a multisubunit DNA-binding protein that binds to all yeast origins of DNA replication in an ATP-dependent manner. The arrangement of an essential sequence combined with redundant, nonessential DNA sequence elements within the *ARS1* replicator represents an unusual structure for a replicator because such a structure has not been described in either bacteria, phage, or eukaryotic virus origins of DNA replication.

To test the generality of the unusual structure of this yeast chromosomal replicator, we have now completed a systematic analysis of another cellular replicator, the *ARS307* locus located on chromosome III in *S. cerevisiae*. Linker scan mutagenesis revealed an essential DNA element (A) containing, like the *ARS1* A element, a perfect match to the degenerate 11-base-pair *ARS* consensus sequence. Additionally, two other important elements were detected and have been designated b1 and b2 (see Fig. 1). Mutations in either of the b1 or b2 elements of *ARS307* reduced origin function, and when mutations were introduced into both elements, origin function was eliminated. Because the general characteristics of the *ARS307* replicator structure resembled the *ARS1* structure, but there was little sequence similarity between them, we have mixed and matched these elements (see Fig. 1). This analysis has revealed that the A and B elements from *ARS307* can substitute for their counterparts in *ARS1*, and vice versa. Moreover, the B1 and B2 elements cannot substitute for each other. Surprisingly, little sequence similarity exists between the B1 ele-

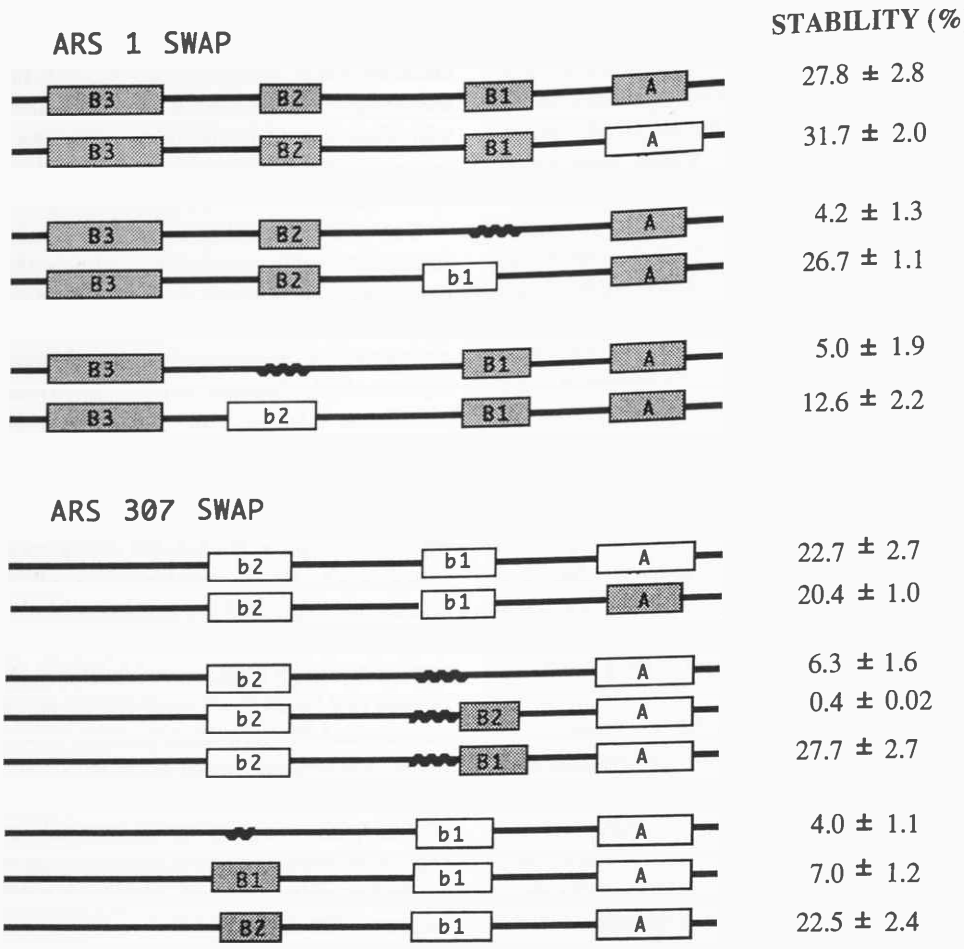


FIGURE 1 Multiple elements in yeast replicator sequences. (*Stippled boxes*) The four elements A, B1, B2, and B3 from *ARS1*; (*open boxes*) the three elements A, b1, and b2 from *ARS307*. The top line in each group shows the wild-type organization for either *ARS1* or *AR307* and the corresponding plasmid stability. The lines below these show the plasmid stability of linker scan mutations (wavy line) or substitutions with heterologous DNA elements.

ments of *ARS1* and *ARS307*, and the only common feature of the B2 elements is a match of 9 bases out of 11 to the degenerate *ARS* consensus sequence. These studies have been very informative because they focus attention on the function of each of these elements, and this clearly presents an immediate challenge.

To understand further the initiation of chromosomal DNA replication, we have focused our attention on the proteins that interact with the *ARS* replicator elements. In last year's Annual Report, we described the purification and characterization of a putative initiator protein, ORC. In collaboration with Ryuji Kobayashi, we have obtained protein sequence from the six core polypeptides that consistently cofractionate with ORC DNA-binding activity and a seventh protein that is variably associated with activity. Using this protein sequence information, the genes for the six core ORC subunits have been cloned from *S. cerevisiae*, and the sequences for most of these genes have been obtained. All are novel proteins that have not been described previously. Most interestingly, one of the genes called *ORC2*, encoding the 72-kD second largest subunit of ORC, is identical to a gene isolated in Jasper Rine's laboratory at University of California, Berkeley, that has not yet been reported in the literature. Margit Foss in Rine's laboratory cloned *ORC2* by com-

plementation of mutants that were defective in silencing a modified silent mating-type gene, *HMR*. The *HMR* gene is part of the complex regulatory apparatus that controls the mating type of yeast. Furthermore, the *orc2* mutants were also temperature-sensitive and arrest with a cell-division-cycle-arrest phenotype consistent with *ORC2* playing an important role in S phase. These observations provide genetic evidence for ORC as a key protein required for cell cycle progression through S phase. Moreover, they suggest that the putative replication initiator protein plays a key role in regulation of gene expression. It is worth noting that studies at Cold Spring Harbor Laboratory approximately 10 years ago by Hicks, Strathern, Klar, and Nasmyth helped elucidate the role of transcriptional silencing in regulation of yeast mating type. In the future, it will be of considerable interest to determine whether these studies on transcriptional silencing and our studies on DNA replication might point to a more general link between DNA replication, transcription control, and chromatin structure.

Chromatin Assembly

G. Bauer, P. Kaufman, N. Kessler, B. Stillman

During S phase in the cell cycle, the DNA present in chromosomes is duplicated along with the chromosomal proteins. Under certain conditions, this process can be duplicated in vitro, with plasmids containing the SV40 origin of DNA replication replicating and being assembled into a chromatin structure that resembles the structure of chromatin in the cell. We have reported previously that a multisubunit protein, chromatin assembly factor-1 (CAF-1), is required for the replication-dependent assembly of histones onto the daughter DNA helices at the replication fork. CAF-1 consists of protein subunits with a relative mass of 150 kD, 60 kD, and 50 kD. In the last year, we have obtained, in collaboration with Ryuji Kobayashi, partial amino acid sequences of these proteins and used this information to clone human cellular cDNAs encoding each protein. Although each protein sequence is novel, the two smaller subunits are related to each other because they contain multiple copies of an approximately 40-amino-acid repeat found in the β subunits of receptor-linked G proteins and a number of other proteins. Interestingly, some of these other proteins are transcription factors that may influence gene expression by altering chromatin structure. Although the function of the repeats is not known, we should be in a strong position to investigate further the individual CAF-1 subunits and the role of the repeats in chromatin assembly by expressing the protein using expression vectors.

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MOLECULAR BIOLOGY OF BOVINE PAPILLOMAVIRUS

A. Stenlund M. Ustav, P. Szymanski, J. Sedman, R. Kluttig, E. Ustav, L. Gandhi, T. Sedman

The papillomaviruses infect and transform the basal epithelium in their hosts, inducing abnormal proliferation of the cells at the site of infection. The resulting tumors (warts) are in most cases benign and will usually regress after some time, but certain types of human papillomaviruses (HPVs 16, 18, 31, 33, and 39) give rise to tumors that are prone to progress toward malignancy, especially frequently cervical carcinoma. In total, HPV DNA can now be found in approximately 80% of the biopsies from cervical carcinomas. Interestingly, in this type of lesion, the papillomavirus DNA is generally found integrated in the host genome, which indicates that viral DNA replication might have some special significance in tumorigenesis, since in normal nonmalignant lesions, the viral DNA is maintained as a freely replicating plasmid in the nucleus of the infected cell.

The correlation between papillomavirus infections and human carcinomas has spurred a great deal of research on the biology of these viruses over the past decade. One of the key impediments to this work has been the inability to define an in vitro cell culture system for any human papillomavirus, largely due to the fact that these viruses normally require specialized differentiating cells that only with great difficulty can be generated in tissue culture. The answer to this problem has been to turn to study of related viruses. Study of the animal papillomaviruses generally does not carry the same restrictions. In particular, a bovine papillomavirus (BPV-1) has become the prototype virus for the papillomavirus group, solely for the reason that a convenient cell culture system is available where oncogenic transformation, gene expression, and viral DNA replication can be studied. To date, more than 60 different papillomavirus genomes have been characterized and cloned, and all of these show a remarkable degree of similarity in their genome organization. This originally formed the basis for the assumption that what was learned from one virus could be applied to another. This assumption has largely been verified; most of the phenomena that were originally defined in the BPV system have subsequently been found also in human papillomaviruses. This includes a considerable degree of conservation concerning the replication apparatus.

In previous years, we have reported the development of a short-term replication assay that was designed and used to define the replication apparatus of BPV. This assay has allowed us to initiate a detailed study of the requirements for BPV DNA replication. The two viral polypeptides, E1 (the initiator) and E2 (a viral transcription factor), that are required for replication and also the necessary *cis*-acting elements have been defined using this assay. During the past 2 years, we have concentrated our work on the functional role of these two polypeptides and their interaction with the *ori* sequence. This year, we have taken the first step into an in vitro system to try to explain at the molecular level the events that are required for initiation of DNA replication of papillomaviruses.

One important characteristic of the papillomaviruses is their ability to maintain the viral DNA in a latent state as a low-copy-number nuclear plasmid. This property is likely to require a number of features in addition to DNA replication, including copy number control and an active partitioning system. During the past year, we have begun to develop systems to study these features.

Role of the E2 Polypeptide in DNA Replication

E. Ustav, M. Ustav, P. Szymanski, A. Stenlund

Our previous studies had demonstrated that the viral transcription factor E2 was essential for replication in vivo. Since the minimal Ori contains a low-affinity

binding site for E2, it seemed likely that E2 would function through binding to this site. To determine if the DNA-binding activity of the protein was required for replication, point mutants were generated in conserved motifs in the carboxy-terminal part of E2, and the resulting mutants were tested for their ability to bind to DNA, and subsequently tested also for their ability to dimerize in an in vivo dimerization assay, since dimerization is a prerequisite for DNA binding. Mutants that were capable of dimerization but were defective in their ability to bind DNA were chosen and tested for their ability to support replication. A perfect correlation was observed between DNA-binding ability and ability to support replication. These results indicated clearly the that DNA-binding capacity of E2 was required for DNA replication and that the E2 polypeptide probably was required to bind to the Ori region.

Mutational analysis of the E2-binding site in Ori, with mutations that either increased or decreased the affinity of E2 for the site or deletions of the entire E2-binding site, demonstrated that the binding site was absolutely required for a functional Ori but that E2-binding sites that were severely crippled for binding of E2 in vitro were still functional for replication. Furthermore, replication activity could be restored to a mutant where the entire E2-binding site had been deleted, by insertion of an unrelated high-affinity E2-binding site at a different position in the plasmid. These results indicated that the position and distance of the E2-binding site relative to the E1-binding site were not critical, at least when a high-affinity E2-binding site was used.

We also generated a series of *ori* constructs where we inserted spacer sequences between the E2-binding site and the recognition sequence for E1. Insertion of a 10-bp spacer resulted in loss of *ori* function with a low-affinity E2-binding site, whereas with a high-affinity E2-binding site, replication activity was retained; in fact, the distance between the E1- and E2-binding sites could be increased to at least 1 kb when multimerized high-affinity E2-binding sites were used. Thus, low-affinity sites could function only when located close to the E1-binding site, and high-affinity sites could function at greater distances. These results were consistent with an interaction between the E2 polypeptide and some other component of the replication machinery, resulting in a stabilization of the E2 interaction with a low-affinity binding site.

Interaction between E1 and E2 Proteins at the Origin of Replication

L. Gandhi, A. Stenlund

A simple model to explain the relationship between affinity of the E2-binding site and distance dependence would be that E2 can participate in an interaction with E1 that would serve to stabilize the binding to a low-affinity site. To explore this possibility directly, we expressed and purified both E1 and E2 proteins from *Escherichia coli* to determine if an interaction between the two proteins could be detected at the origin of replication. By using DNase footprint assays, we could determine that the two proteins bind cooperatively to the origin when the binding sites for both proteins are present (Fig. 1). Furthermore, by

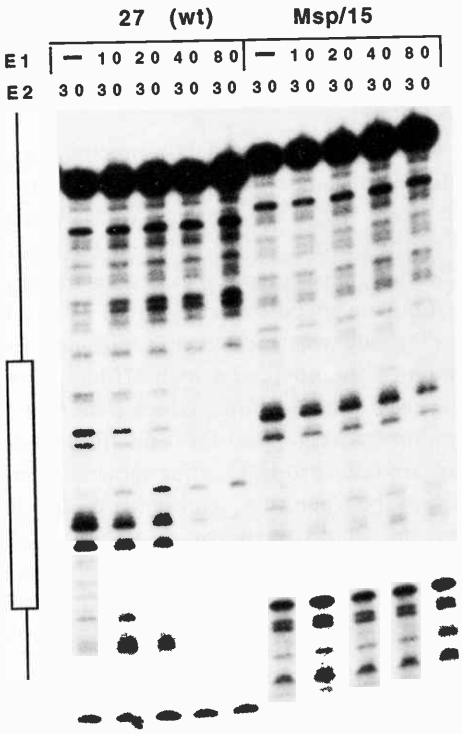

FIGURE 1 E1 and E2 proteins bind cooperatively to the Ori when binding sites for both proteins are present. DNase footprint reactions were carried out on the wild-type Ori (27) and on a mutant Ori that lacks the E2-binding site (Msp/15) using purified E1 and E2 proteins. At the low level of E2 used (30 ng), no footprint is detectable on either template in the absence of E1. The bar indicates the combined E1 and E2 footprints that appear on the wild-type template.

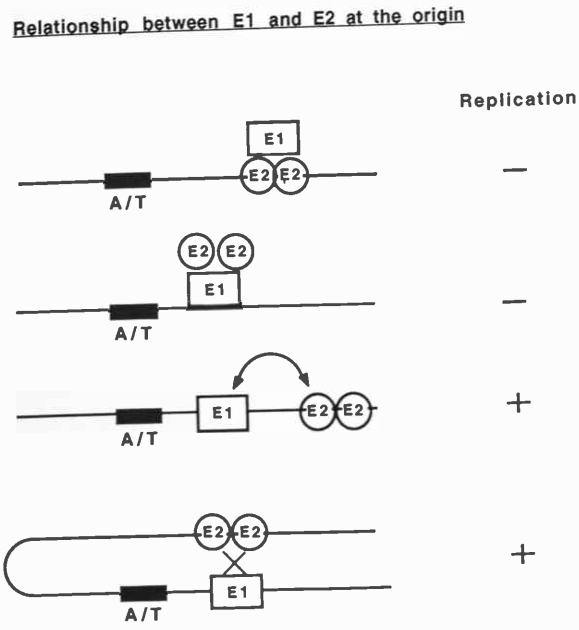

FIGURE 2 A schematic figure illustrating possible arrangements of viral proteins and *cis*-acting sequences for replication of the minimal Ori in vivo.

using a so-called McKay DNA precipitation assay, we could demonstrate that the ability of E1 to bind to its site at the Ori in the presence of E2 was proportional to the affinity of the adjacent E2-binding site for E2. These experiments also demonstrated that E2 could stimulate specific binding of E1 even when the E2-binding site was located at a distance from the E1-binding site, provided a high-affinity E2-binding site was used. These results, which indicated a direct interaction between the E1 and E2 when both proteins are bound to DNA, thus showed a very good correlation between the requirements for in vitro binding and in vivo replication activity. Taken together, these results indicate that a specific ternary complex composed of E1, E2, and the Ori DNA is required for functional initiation of replication in vivo (Fig. 2).

Replication In Vitro

J. Sedman, A. Stenlund

To further investigate the molecular events that are required for replication of BPV DNA, we have started experiments to characterize the initiation of DNA replication using a cell-free system. An in vitro replication system for BPV has recently been developed by M. Botchan and colleagues (University of California, Berkeley). Using this system (which is based on a crude nuclear extract from mouse FM 3A cells), we can achieve replication of the BPV Ori if the E1 protein, which is expressed and purified from *E. coli*, is added. Interestingly, this system shows distinctive differences compared to replication in vivo. Although replication in vivo shows an absolute requirement for the E2 protein and E2-binding sites in *cis*, in the in vitro replication system, E2 is not absolutely required but has a stimulatory effect on replication. However, this stimulatory effect does not appear to be dependent on the presence of binding sites for E2. We are presently trying to determine the basis for this difference between in vivo and in vitro replication.

Maintenance Replication

M. Ustav, A. Stenlund

A unique and interesting aspect of BPV replication is the ability of the viral genome to persist in BPV-transformed cells by replicating as a nuclear plasmid with a constant copy number under cell cycle control. Last year, we described a system to study this particular aspect of BPV replication which has been termed maintenance. Our strategy has been to develop cell lines that express the E1 and E2 polypeptides constitutively and allow replication from the BPV origin of replication (these cells are the functional equivalent of COS cells for SV40). To assay for maintenance, a vector containing a *neo* resistance marker and an origin-containing fragment of the BPV genome is transfected into this cell line. After selection for G418-resistant colonies and further passage for several weeks, DNA can be prepared and analyzed for the presence of episomal vector sequences. These experiments demonstrated that the E1 and E2 gene products were the only viral proteins required for maintenance. The next question that we addressed was whether the *cis*-acting sequences that were sufficient for short-term replication (i.e., the minimal Ori) were also sufficient for maintenance. These experiments were carried out by inserting fragments from the BPV genome of increasing size all containing the

minimal Ori into the *neo* plasmid. The Ori constructs were tested initially in a short-term replication assay in which all constructs replicated to similar levels. After G418 selection and passaging, however, the minimal Ori constructs could not be recovered as episomal DNA. These results indicated that additional sequences were required for stable maintenance of BPV. Further mapping of these sequences indicates that they are located within the E2-dependent transcriptional enhancer.

Regulation of Viral DNA Replication

P. Szymanski, A. Stenlund

The E2 open reading frame of BPV encodes, in addition to the full-length polypeptide that functions as a *trans*-activator, two shorter polypeptides that contain the same carboxy-terminal DNA-binding/dimerization domain as the full-length protein. These two polypeptides have been shown to act as competitive repressors of transcription from E2 responsive promoters; however, the mechanism of action has not been determined. Two obvious possibilities are either competition for DNA binding, since these three proteins have the same DNA-binding specificity, or formation of inactive heterodimers, since the dimerization domain is also shared. Since the full-length E2 is also involved directly in DNA replication, we were interested in determining if the short forms of E2 could affect DNA replication directly, and what relative contributions heterodimerization versus competition for binding make to this repression.

To distinguish between these two mechanisms, we utilized several point mutants in the carboxyl terminus of E2 that are deficient for DNA binding but are still capable of dimerization. If either of the short forms of E2 is included in an E2-dependent replication assay, a low-level repression of replication is observed. If instead a DNA-binding-deficient mutant version of the short E2 is used, repression is increased considerably. In contrast, repression of transcription behaves differently: The wild-type version of the short E2 is a considerably better repressor than the DNA-binding-deficient mutant of the same protein. In both assays, however, the most effective repressor is a DNA-binding-deficient form of full-length E2 that can efficiently repress both replication and transcription. The conclusion from these experiments is that hybrid dimers, formed between the wild-type activator and the DNA-binding-deficient mutant, are incapable of activating transcription due to a lack of DNA-binding activity of the hybrid. In contrast, hybrid dimers, consisting of the wild-type activator and the short form of E2 which are competent for DNA binding, appear to be capable of supporting replication to some extent but are not functional for transcription. Taken together, these results indicate that the naturally occurring repressor forms of E2 function largely through competition for DNA binding with the full-length E2 polypeptide.

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ADENOVIRUS TRANSFORMING FUNCTIONS

E. Moran S. Abraham, A. Ebrahim, Y. Rikitake, H.-G.H. Wang, P. Yaciuk, F. Chadrin, A. Pepe, N. Dawkins, J. Altman

The adenovirus E1A gene products continue to serve as extremely sensitive genetic probes for dissecting the pathways regulating cell growth. Within the past few years, the recognition that one host-cell growth-regulating function in E1A is genetically linked with activation of the cellular E2F transcription factor, and with binding of a family of cellular products including the growth suppressor gene, pRB, has led to the understanding that E2F is an important regulator of cell-cycle-specific transcription and that pRB, its homologs, and their associated cyclins are cellular modulators of E2F activity.

Our interest in understanding the means by which cell growth control pathways can be overridden leads us to study not just the growth-regulating mechanisms linked with the pRB-binding function of E1A, but also the control points at which the E1A products can influence cell growth independently of the pRB-binding function. In our laboratory during the past year, we have continued to characterize another major cell-growth-regulating region of E1A and a cellular protein, p300, that is bound by E1A at this site. Residues important for p300 binding are required for the transformation function of E1A and are involved in other E1A-mediated gene-regulating functions, including activation of cell-cycle-regulated products and repression of tissue-specific enhancer activity. In the past year, we have significantly refined our understanding of the E1A active sites and the distinction between the binding sites for p300 and the pRB-related proteins. We have also discovered that these associations occur in the same E1A complexes, and probably on the same E1A molecule, bringing pRB and the pRB-related proteins and their associated cyclins and kinases into close proximity with p300. Interestingly, the E1A products of the oncogenic serotype, adenovirus 12 (Ad12), are able to inactivate the p300-binding site via a posttranslational modification, leaving a stable E1A product able to bind pRB, but not p300. In the past year, we have also increased significantly our understanding of the nature and function of p300 itself. We have succeeded in developing a series of p300-specific monoclonal antibodies, which have served to reveal two distinct forms of p300, and the existence of a number of p300-associated cellular proteins. Genetic analysis now suggests that p300 may have specific interactions with the basal transcription assemblage: The ability of the E1A products to activate the hsp70 promoter through specific TATA box sequences has been linked with E1A sequences required for p300 binding, and we have obtained physical evidence of an interaction between p300 and the basal transcription factor, TBP (the TATA-box-binding protein). These results are described in more detail in the sections below.

Analysis by Mutation and Monoclonal Antibody Competition Reveals the Independence of the E1A-binding Sites for p300 and the pRB-related Proteins

H.-G.H. Wang, Y. Rikitake, P. Yaciuk, S. Abraham, F. Chadrin, N. Dawkins, J. Altman, E. Moran

Our current understanding of the E1A regions involved in cell growth control is illustrated in Figure 1. Although conserved region 1 has been known for some time to be required for the binding of most E1A-associated proteins, our recent analysis has indicated that region 1 probably is composed of two subregions with distinct binding activities. Through a combination of site-specific mutation and analysis of monoclonal antibody competition for cellular protein-binding sites on E1A, we have obtained strong evidence that p300 and the pRB-related proteins bind within conserved region 1 at nonoverlapping sites. This study has also revealed specific amino acid residues in the amino-terminal region that are required for p300 binding and E1A-mediated changes in cell growth patterns. We have also found that the rat and human pRB molecules have distinguishable requirements for binding sites on the E1A proteins. This increasingly clear knowledge of the structure of the E1A-binding sites has significantly expanded our ability to design and interpret E1A mutant studies.

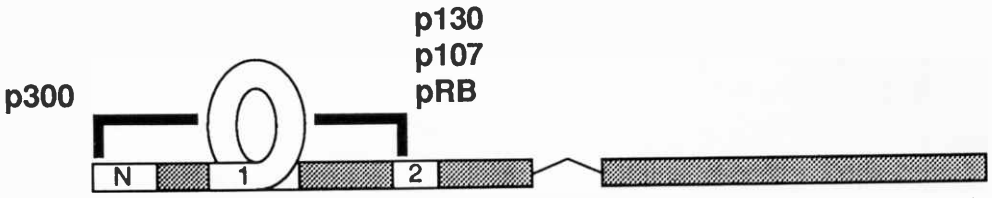

FIGURE 1 Schematic model of E1A functional regions directed at cell cycle control mechanisms and of the cellular proteins bound by each. Adenovirus E1A oncogene-encoded sequences essential for transformation and cell-cycle-regulating activities are localized at the amino(N)-terminus and in regions of highly conserved amino acid sequence designated conserved regions 1 and 2. These regions interact to form the binding sites for two classes of cellular proteins: the 105-kD retinoblastoma gene product and its possible homologs, whose association with the E1A products is specifically dependent on region 2, and another class that so far in vivo is known to include only a large cellular DNA-binding protein, p300, whose association with the E1A products is specifically dependent on the amino-terminal region. Association between the E1A products and either class of cellular proteins can be disrupted by mutations in conserved region 1. A combination of site-directed point mutagenesis and monoclonal antibody competition experiments now suggests that p300 and pRB bind to distinct, nonoverlapping subregions within conserved region 1.

The Binding Sites for p300 and the pRB-related Proteins Are Apparently Occupied on the Same E1A Molecule

P. Yaciuk, H.-G.H. Wang, F. Chadrin, A. Pepe, N. Dawkins, E. Moran

Our ongoing studies of the E1A-binding sites have also resulted in the surprising discovery that p300 and the region-2-associated proteins are in the same E1A complex and can probably occupy the same E1A molecule. The availability of a strong panel of p300-specific antibodies, reported in more detail below, has permitted us to examine E1A complexes from the viewpoint of p300. p300-specific immunocomplexes clearly contain protein species that comigrate with the E1A region-2-associated proteins, p130, p107, and p105-RB (Fig. 2). Comparison by partial proteolytic peptide mapping confirms that these are indeed the same protein species. Several lines of evidence suggest that the amino-terminal and region-2-binding sites are occupied on the same molecule as opposed to different molecules within E1A oligomeric complexes: First, p300-specific antibodies cannot bring down any of the region-2-associated proteins in a mixed infection where region-2 and the amino-terminal binding site are each present, but on separate molecules. Second, all attempts to show co-immunoprecipitation between different E1A molecules in these complexes have proved negative. Third, although the amino-terminal and region-2-binding sites can function independently, there is some evidence that binding at one site stabilizes binding at the other. (Binding of p107 is lost if specific single residues within region 1 and region 2 are altered simultaneously but appears unaffected when only one is altered. Nevertheless, disruption of the p300-binding site in combination with just the region-2 substitution abrogates p107 binding, suggesting that when p107 binding is accomplished through just a single site, binding of p300 serves to stabilize the association.) Together, these observations suggest strongly that the region-2-binding site and the p300-binding site are generally both occupied on the same E1A molecule.

The ability of the E1A products to bring the region-2-associated proteins into close proximity to p300 may have important implications for the function of the E1A complexes. A major part of the E1A population appears to be bound to p300 in E1A-expressing cells, and our preliminary studies have suggested that a large part of p300 is bound to cellular DNA, even in the presence of E1A. The presence among the region-2-associated proteins of species that are themselves associated with cellular cyclins and kinases raises the possibility that these species may have important interactions not previously recognized. Thus, it will be important to determine the cellular location of the E1A complex and the functional consequences of the structure of the E1A complexes.

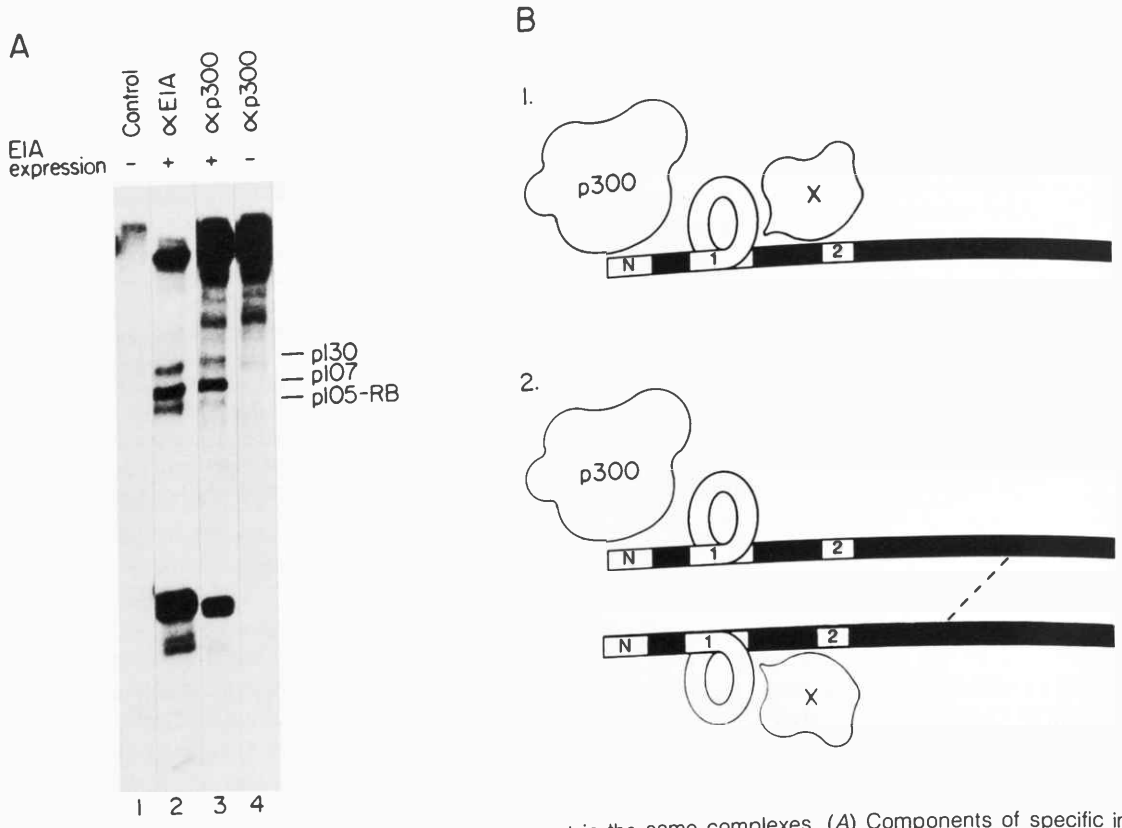

FIGURE 2 p300 and the pRB-related proteins are present in the same complexes. (*A*) Components of specific im munocomplexes, separated on SDS-polyacrylamide gels. E1A-specific antibodies (lane *2*) will precipitate the E1A products and the E1A-associated proteins, p300, p130, p107, and p105-RB (the positions of each of these proteins in the gel is indicated at the right). p300-specific antibodies (lane *3*) will precipitate the same group of proteins, indicating that p300-bound E1A complexes are not distinct from E1A complexes containing any of the pRB-related proteins. In the absence of E1A expression, the p300-specific antibodies (lane *4*) do not coprecipitate the pRB-related proteins, indicating that the p300-specific antibodies do not recognize these species directly, and only pull them down when they are in association with the E1A products. Although these results are compatible with either of the models shown in panel *B*, additional evidence cited in the text favors model number 1, the situation where both binding sites are occupied on the same E1A molecule, as opposed to model 2, where the cellular proteins bind to different E1A molecules, which are themselves associated in oligomeric complexes (indicated by the dashed line).

The Oncogenic Serotype, Ad12, Generates Stable, Prominent Forms Able to Bind pRB but Not p300

H.-G.H. Wang, P. Yaciuk, E. Moran [in collaboration with R.P. Ricciardi, University of Pennsylvania, M. Green, St. Louis University Medical Center, and K. Yokoyama, Tsukuba Life Science Center, Japan

The RB product binds to E1A sequences that are highly conserved among the E1A products of various serotypes, whereas p300 binding requires sequences at the E1A amino terminus, a region not highly conserved. To help evaluate the roles of the E1A-associated proteins in cell growth control, we compared the p300-binding abilities of the E1A products of Ad5 and the more oncogenic Ad12 serotypes. We have found that in spite of encoding a sequence that varies somewhat from the p300-binding sequences of Ad5 E1A, the Ad12 E1A products associate with p300 with an affinity similar to that of the Ad5 E1A products. Both the 12S and 13S splice products of Ad12 E1A, like those of Ad5 E1A, encode proteins able to associate with p300. In addition, like Ad5 E1A, Ad12 E1A can bind p300 and the region-2-associated proteins, such as pRB, in the same complex. Interestingly, though, the proteins translated

from both Ad12 E1A splice products also give rise to prominent amino-terminally modified forms that are unable to associate with p300. This modification, at least in the 13S product, does not appear to diminish the affinity of this product for pRB. The generation in this serotype of a stable modified E1A form that specifically sheds the p300-binding site adds strong support to our hypothesis that there are functional consequences to binding p300 and the region-2-associated proteins in the same complex.

Development of p300-specific Monoclonal Antibodies Reveals the Presence of Two Prominent p300-related Species and Two Distinct Categories of Monoclonal Antibodies

P. Yaciuk, A. Pepe, E. Moran

The targeting of p300 by E1A implies strongly that p300 plays a significant role in cell-growth-regulating processes. However, our previous analysis of p300 expression and phosphorylation revealed little difference in p300 as visualized from cells at various different stages of cell growth. An analogy with the most characterized E1A-associated protein, pRB, suggests that p300 may participate in regulated processes via specific protein-protein associations. Since these kinds of interactions are often excluded from polyclonal serum-derived immunocomplexes, we set out to generate monoclonal antibodies that might be more useful in visualizing E1A-associated proteins. This endeavor resulted in the generation of 11 monoclonal lines, designated NM1 through NM11.

One of the earliest observations to arise from analysis of p300 with these antibodies is that a subset of them also immunoprecipitate a prominent product of about 270 kD that shows a strong relationship to p300. The antibodies can be separated into two categories distinguished by whether or not they immunoprecipitate the 270-kD form (Fig. 3A). The close relationship between the 270-kD species and p300 itself is indicated by the fact that both species are recognized directly by the monoclonal antibodies. In addition, these species show closely related partial proteolytic peptide patterns. The prominent appearance of the 270-kD species was a bit surprising because, although the species seen in association with

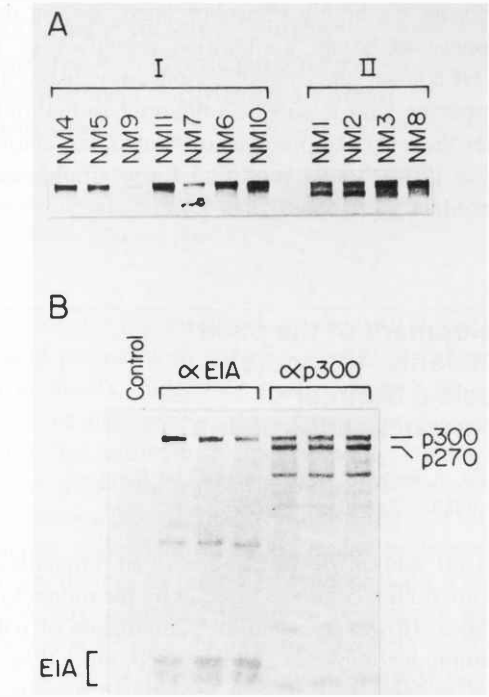

FIGURE 3 Development of p300-specific monoclonal antibodies reveals the presence of two prominent p300-related species and two distinct categories of monoclonal antibodies. Eleven p300-specific monoclonal antibody lines (NM1 through NM11) were generated in this project. Interestingly, these fall into two categories, shown in panel *A*. Category I, which includes 7 of the 11, recognizes predominantly a single form of p300 that comigrates with the major form seen in E1A-specific immunocomplexes (see panel *B*). Category II consistently immunoprecipitates two p300-related forms, of approximately 300 and 270 kD. In panel *B*, E1A-specific immunocomplexes are compared with immunocomplexes from a category II monoclonal line, in triplicate. It is clear that the E1A products associate preferentially with the p300 form. The p270 form is recognized directly by the antibodies, and its partial proteolytic digest pattern is very similar to that of the p300 form, indicating that these are closely related species. It will be important to determine whether they are differentially modified forms of the same gene product or the distinct products of two closely related genes.

E1A often appear to be heterogeneous, forms comigrating with the 270-kD species have not appeared prominently. Thus, this form, although apparently abundant in cells, does not seem to associate readily with the E1A products. This distinction is readily apparent when E1A-specific immunoprecipitations are compared side-by-side with immunoprecipitations done with a monoclonal line that recognizes both the p300 and p270 forms (Fig. 3B). Both the p300 and

p270 forms are highly phosphorylated, so one does not appear to be an unmodified precursor of the other. Of course, other modifications may distinguish these species, and it will be important to determine whether these are the products of related, but distinct, genes or differentially modified forms of the same gene product.

Development of the p300 Monoclonal Antibodies Reveals a Number of p300-associated Proteins

P. Yaciuk, A. Pepe, S. Abraham, H.-G.H. Wang, F. Chadrin, E. Moran

The p300 and p270 forms described immediately above are both recognized directly by the monoclonal antibodies. However, when all components of p300/p270 immunocomplexes are examined, more than ten cellular proteins that are not recognized directly by the antibodies are revealed. Curiously, these additional species appear only in immunocomplexes with the category of antibody that recognizes the p270 form in addition to the p300 form. The evidence that these are not cross-reactive species and that they are lost from the immunocomplexes under conditions that disrupt protein-protein associations indicates strongly that these are specific p300/p270-associated proteins. Most of the associated species, like the region-2-associated proteins and p300/p270 and the E1A products themselves, appear to be phosphoproteins. The phosphoprotein forms are shown in Figure 4. The selective appearance of the associated products only in immunoprecipitations that contain p270 in addition to p300 could be interpreted to indicate that these factors are associated specifically with p270. Alternatively, both p300 and p270 may associate with cellular factors, but the epitope specificity of the first antibody category may be such that it is incompatible with the binding of the associated proteins; this epitope may be less accessible on the p270 form. Work is in progress to distinguish these possibilities, which will be important in understanding the functional consequences of p300/p270 cellular complex formation and the effect of E1A expression on these complexes. The ability to visualize these proteins will now enable us to go on to determine whether there are associations that are specific to individual stages of cell growth, such as phases of the cell cycle, transformed versus normal cells, or aging and terminally differentiated cells.

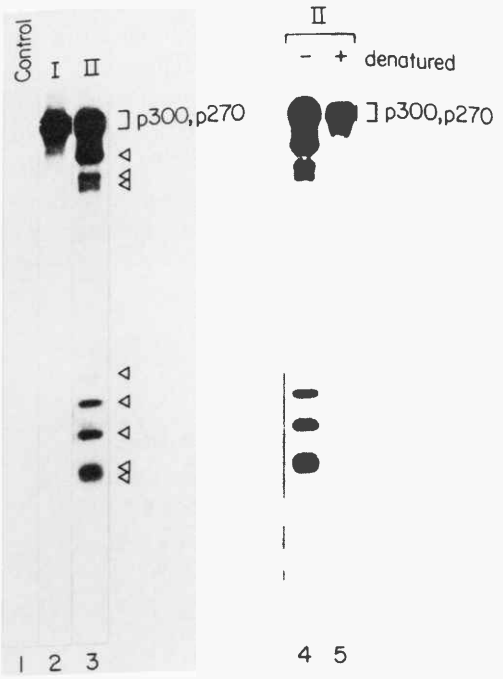

FIGURE 4 The p300/p270 products associate with a number of cellular proteins, many of which appear to be phosphoproteins. When the total components of the p300/p270 immunocomplexes are examined, a far more dramatic difference becomes apparent between the two categories of antibodies distinguished above. The category I antibodies coprecipitate few if any cellular proteins (a representative is shown in lane *2*), whereas the category II antibodies coprecipitate a number of cellular proteins (shown in lane *3*). Shown here are the species visible via ^{32}P labeling. We consistently see three relatively high-molecular-mass species of approximately 190, 170, and 160 kD, and five lower-molecular-mass species of approximately 64, 59, 50, 45, and 44 kD. These are indicated by arrows to the right of lane *3*. ^{35}S labeling reveals a similar pattern, with two additional prominent species of about 75 and 71 kD. None of the proteins designated as associated species are recognized directly by these antibodies. When the immunocomplexes (lane *4*) are denatured to disrupt protein-protein associations, then probed again with the same antibody, only the p300 and p270 forms reappear (lane *5*).

An important property already revealed from our study of these complexes is that p300 and at least two of the p300-associated phosphoprotein species are also present in specific complexes with the general transcription factor, TBP (the TATA-box-binding protein). These results are described further below.

Genetic Data Implicate the Amino-terminal E1A Function in *trans*-Activation via Specific TATA Box Sequences

E. Moran, F. Chadrin [in collaboration with V. Krauss and J. Nevins, Duke University]

Several years ago, it was observed by J. Nevins and others that the 12S E1A splice product can activate the hsp70 promoter dependent on specific TATA box sequences. The E1A 12S product can activate a wild-type hsp70 promoter with its cognate TATA box element, TATAA, but cannot activate a mutant hsp70 promoter in which the SV40 TATTTAT sequence has been substituted for the normal hsp70 TATAA element, even though the heterologous element is still activated in response to heat shock. In the past year, in a continuing collaboration with the Nevins laboratory, we have supplied newly generated E1A mutants with amino-terminal alterations that have helped to map this activity in E1A. These mutants indicate that the ability of E1A to *trans*-activate via the hsp70 TATA element is dependent on E1A sequences also involved in p300 binding and independent of region-2-specific associations.

Our previous results have indicated that p300 is a sequence-specific DNA-binding protein and thus is likely to be involved in transcription. However, p300 shows no affinity for sequences related to TATA box elements, showing instead a specific preference for GC-rich sequences related to certain tissue-specific enhancer elements known to be targeted by E1A in a manner dependent on the p300-binding region (described in 1991 Annual Report). These properties of p300 and the E1A products appear to be linked to the ability of the E1A products to repress many forms of tissue-specific gene expression. Whether they are also linked to the ability of the E1A amino-terminal active site to activate hsp70 and cell-cycle-specific gene expression remains to be determined. However, the link revealed by E1A mutant anaylsis between p300 association and TATA box responsiveness raises the possibility that p300, even if bound to upstream GC-rich elements, may have specific interactions with the protein complexes formed at the TATA box. Evidence that this indeed is so is described in the following section.

p300 and p300-associated Proteins Are Components of Specific TATA-binding Protein Complexes

S. Abraham, P. Yaciuk, H.-G.H. Wang, A. Pepe, E. Moran [in collaboration with S. Lobo, Cold Spring Harbor Laboratory]

Last year, we reported evidence that p300 is a DNA-binding protein with specific affinity for known enhancer motifs, suggesting that p300 may be a component of transcription factor complexes. The possibility that upstream element binding factors might interact with basal transcription factors, coupled with the evidence described above indicating that the E1A products, dependent on p300-binding sequences, can activate the hsp70 promoter dependent on a specific TATA box sequence, led us to investigate whether p300 interacts, directly or indirectly, with the TATA-binding protein (TBP). The availability of the panel of p300-specific monoclonal antibodies developed in our laboratory, together with a panel of TBP-specific monoclonal antibodies developed by S. Lobo in the Hernandez laboratory (see Genetics Section), enabled us to investigate this possibility through the analysis of specific immunocomplexes. We found indeed that TBP-specific immunoprecipitations show a 300-kD protein copre-cipitating with TBP. This protein is lost from the precipitated material if the lysates are boiled in SDS prior to immunoprecipitation, implying that its presence does not result from nonspecific antibody cross-reactivity but is dependent on specific association with TBP (these interactions are shown in Fig. 5). The TBP-associated 300-kD protein and p300 originally defined by E1A association show indistinguishable partial proteolytic digest patterns, indicating that these are identical or closely related species. Moreover, p300-specific complexes and TBP-specific complexes include at least two additional common polypeptide species, phosphoproteins of 64 kD and 59 kD.

These results suggest that p300 interacts with TBP, probably through intermediate protein-protein associations. Additionally, the observation that the TBP-specific immunocomplexes examined here include both the p270 and p300 forms indicates that both of these forms can participate in cellular complex formation.

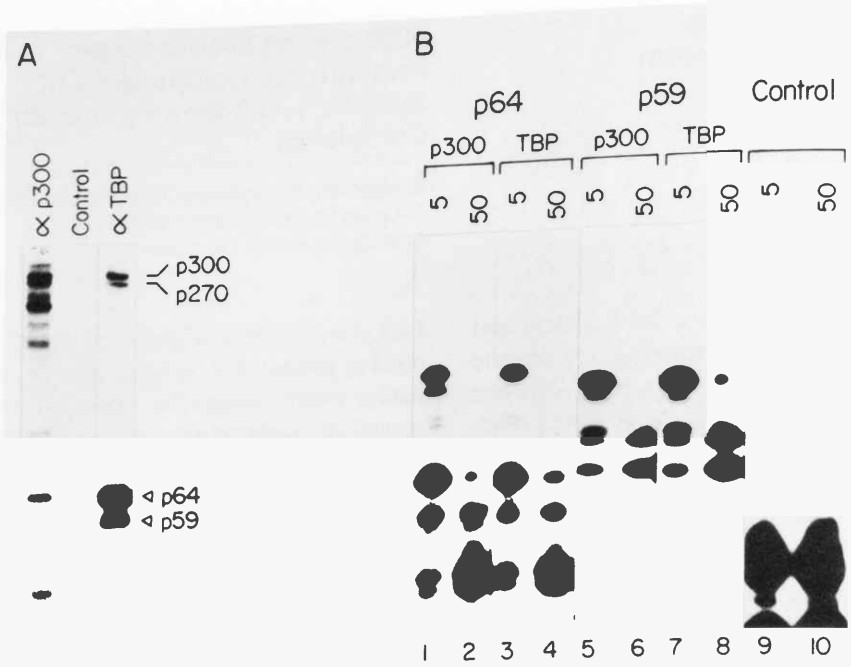

FIGURE 5 Evidence for specific physical interactions between p300 and TBP (TATA-binding protein) complexes. A subset of TBP-specific monoclonal antibodies will coprecipitate p300 in immunocomplexes. One of these is shown in panel *A*, lane *3*, where the TBP-specific immunocomplex is compared with a p300-specific immunocomplex (lane *1*). (These lysates are labeled with ^{32}P to facilitate detection of the p300-associated phosphoproteins and thus do not reveal TBP, which is not known to be phosphorylated.) The suggestion that these immunocomplexes represent authentic in vivo protein associations is supported strongly by the observation that other p300-associated proteins, the phosphoprotein species, p64 and p59 (marked with arrows in panel *A*), appear in immunocomplexes with the same subset of TBP-specific monoclonal antibody lines that coprecipitate p300. That these are indeed the same species is indicated by their essentially identical peptide digest patterns, shown in panel *B*. The p64 species, derived from either p300-specific or TBP-specific immunocomplexes, show the same pattern (lanes *1* and *2* compared with lanes *3* and *4*), as do the p59 species from either immunocomplex (lanes *5* through *8*). A control protein of similar size shows a very different pattern (lanes *9* and *10*).

Conclusions and Future Plans

P. Yaciuk, H.-G.H. Wang, A. Ebrahim, F. Chadrin, J. Altman, E. Moran

This year has seen considerable progress both in characterizing the intracellular interactions of p300 and in understanding the molecular formation of cellular protein complexes on the E1A molecule. The observation that p300 and p300-associated proteins are present in TBP-specific complexes strongly supports previous indications that p300 plays a significant role in cellular transcription. The visualization of p300-associated cellular factors is an important step toward identifying and understanding the cellular role of p300 and the regulatory pathways in which it functions. Increased understanding of the nature of E1A complex formation has revealed a potential for direct interaction between p300 and the region-2-associated proteins that may be very important in understanding the means by which the E1A products override cell growth control mechanisms, thus revealing vulnerable cell growth control points.

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PROTEIN CHEMISTRY

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The Protein Chemistry laboratory conducts research on signal transduction and cell growth using advanced methods of analytical and preparative biochemistry. These methods include automated protein sequence analysis, high-performance liquid chromatography (HPLC), amino acid analysis, preparative electrophoresis, mass spectrometry, and peptide synthesis. Using such procedures allows us to deduce chemical structures of proteins and to synthesize structural and functional domains. In addition, physical analysis of proteins by mass spectrometry, in conjunction with chemical studies, permits the determination of posttranslational modifications of proteins, such as phosphorylation and acylation. Our goal is to attack biological problems of the control of cell growth by using an arsenal of state-of-the-art biochemical methods. Often, this requires the development of new methods when existing procedures are not adequate to solve the cell biological question. This chemical approach to cell biology is highly complementary to genetic approaches in many other laboratories at Cold Spring Harbor, and analytical biochemistry is an essential part of a multidisciplinary approach to problems of normal and abnormal cell growth, as in cancer.

Synthetic Peptide Substrates for Protein Kinases

D.R. Marshak, M. Vandenberg, I.J. Yu, F. Sun, M. Meneilly, G. Binns

Protein kinases are enzymes that catalyze the transfer of phosphates from the γ-phosphate position of ATP to a hydroxyl moiety (serine, threonine, or tyrosine) on proteins. The recognition sequences for protein kinases are surprisingly short; usually only four to six amino acid residues in linear sequence are required for specifying the substrate. Therefore, synthetic peptide substrates have been quite useful as model substrates for protein kinases. Such peptides, usually 10–20 residues in length, can be synthesized by solid-phase methods using automated instrumentation. They are constructed as protected molecules on polystyrene supports and then deprotected and cleaved from the support by treatment with acid. In

our laboratory, rigorous purification and characterization are carried out to assure purity and identity of the material. We have documented evidence that products of the side reactions of peptide synthesis can alter the measured kinetic properties of the substrate (Marshak and Carroll, *Methods Enzymol. 200:* 134 [1991]). Therefore, we use mass spectrometric measurements to assess the molecular weight of the product to eliminate unwanted modified side products that arise from incomplete removal of protecting groups or other modifications. Combined with HPLC and amino acid analysis, mass spectrometry has allowed us to prepare peptides with excellent characteristics as kinase substrates, including low K_M values.

During the past year, we have examined the substrate specificity of $p34^{cdc2}$ isolated from HeLa cells arrested during mitosis by the drug nocodazole. This compound disrupts microtubules and thus does not allow a cell to pass metaphase because there is not a functional mitotic spindle apparatus for chromosome segregation. These mitotic human cells have very high levels of $p34^{cdc2}$ complexed with cyclin B. This enzyme has been thought to have the substrate specificity Ser/Thr-Pro-$(X)_n$-Z, where the phosphorylated residue can be either a serine or threonine that is always followed by proline. The residues marked X are usually polar, where n = 1–3, and Z represents a basic residue. We tested the requirement for the basic residues downstream in a series of synthetic substrates based on the $p34^{cdc2}$ phosphorylation site found in SV40 T antigen. By changing basic residues to leucine hydrophobic residues, we were able to alter the K_M, the V_{max}, or both, depending on the positions of the residues substituted. Furthermore, we altered the polar uncharged residue X to a negatively charged residue, glutamic acid, and observed increased phosphorylation of that substrate. These data are significant because new, putative sites of phosphorylation by $p34^{cdc2}$ might be due to a related enzyme with slightly altered substrate specificity, such as $p33^{cdk2}$ or $p34^{cdc2}$ bound to a different cyclin molecule.

Molecular Cloning and Expression of Casein Kinase II Subunits

N. Chester, I.J. Yu, M.T. Vandenberg, D.R. Marshak

The enzyme casein kinase II (CKII) is a protein serine/threonine kinase found in all eukaryotic cells. Its ubiquitous distribution among species and tissues implies a function central to all nucleated cells. The enzyme consists of two subunits, α and β, with molecular mass ranges of 37–44 kD and 24–28 kD, respectively, and an apparent subunit composition of $\alpha_2\beta_2$. The α subunit is found in two different forms, known as α and α′, that arise from separate genes. To answer questions about the mechanism of enzyme regulation, we have cloned and expressed DNA molecules coding for the full-length forms of the α and β subunits. To do this, a novel method using the polymerase chain reaction was developed (N. Chester and D.R. Marshak, in press).

We have also obtained a clone of the α′ subunit from Dr. Ed Krebs in Seattle. We built these clones into a T7 expression vector (a gift from W. Studier, Brookhaven, and J. Kuret, CSHL) for production of the subunits of the protein. In addition, the α′ subunit was expressed as a fusion protein with the bacterial maltose-binding protein. This construction permits rapid purification of the fusion protein on affinity columns, followed by cleavage of the enzyme from the binding protein. M. Vandenberg has shown that the purified recombinant α′ subunit can be activated in vitro by the recombinant β subunit. We plan to go on with large-scale expression of various forms of the enzyme for structural analysis by X-ray diffraction.

Cell Cycle Regulation of Casein Kinase II

G.L. Russo, I.J. Yu, G. Binns, M. Vandenberg, D.R. Marshak

A set of studies has been conducted by Gian Luigi Russo to investigate the cell cycle regulation of the phosphorylation of $p34^{cdc2}$. This enzyme has major phosphorylation sites at threonine and tyrosine residues that regulate its activity positively and negatively, respectively. Previous reports of serine phosphorylation have led us to attempt to identify these minor sites of phosphate attachment. We discovered that the $p34^{cdc2}$ kinase, which is central to growth control in eukaryotic organisms, is phosphorylated on Ser-39 during the G_1 phase of the HeLa cell division cycle (Russo et al. 1992). This site was mapped on protein labeled in vivo and in vitro. We utilized our new phosphoprotein sequencing methods to identify the exact site of phosphorylation. The en-

zyme responsible for this phosphorylation event is casein kinase II, which was previously shown by us to be activated during middle to late G_1. This phosphorylation was demonstrated using purified native $p34^{cdc2}$, recombinant protein from *Escherichia coli*, and representative synthetic peptides. Different phosphorylated forms of $p34^{cdc2}$ were identified on two-dimensional gels (in collaboration with R. Franza).

Subcellular Localization of Casein Kinase II

N. Chester, I.J. Yu, D.R. Marshak

The CKII enzyme activity is found both in cytosol and in nuclei, and there are substrates identified in both locations. Cytosolic substrates include proteins involved in translational control (eIF-2, -3, -4B, -5), metabolic regulation (glycogen synthase), and the cytoskeleton (nonmuscle myosin heavy chain, β-tubulin). Substrates found in the nucleus include DNA topoisomerase II, RNA polymerases I and II, oncoproteins such as Myc, Myb, and SV40 large T antigen, and transcription factors such as serum response factor. The extraordinary range of substrates for this enzyme supports the contention that CKII plays a significant role in cell physiology. We have developed a large set of specific antibodies to synthetic peptide antigens that react with the individual subunits of CKII. These antibodies allowed us to continue our studies of the subcellular localization of the subunits of the enzyme during the cell division cycle of HeLa cells. Using immunofluorescence microscopy, we examined asynchronous populations of cells, as well as cells arrested and released from the G_1/S transition by the chemical hydroxyurea. The results indicated that the α and β subunits are localized to the cytoplasm during interphase and throughout the cell during mitosis. However, using an antibody with different specificities, it appears that a pool of α subunit does exist in the nucleus. The reactivities of the epitope or the physical localization of the enzyme itself changes at the G_1/S-phase transition. These dynamics suggest that CKII may affect cell division cycle control by altering its conformation or localization to different subcellular compartments.

Structural Analysis and Phosphorylation of Nuclear Proteins

D.R. Marshak, M. Vandenberg, G. Binns [in collaboration with T. Curran, Roche Institute; M. Mathews and A. Krainer, Cold Spring Harbor Laboratory; and E. Harlow, Massachusetts General Hospital]

DAI KINASE

Significant progress has been made on mapping the autophosphorylation sites on the double-stranded RNA-activated protein kinase, DAI, done in collaboration with M. Mathews. The kinase is phosphorylated and immunoprecipitated on a large scale. The protein is eluted from the precipitate in formic acid, digested with cyanogen bromide, and subjected to reverse-phase chromatography. At least three peptides have been recovered from these digests. Using phosphoprotein sequencing methods, we have tentatively identified one site. Further subdigestion of the other fragments using proteases has enabled us to recover other phosphorylated peptides for analysis.

SPLICING FACTOR 2

We have been trying to determine the in vivo phosphorylation sites of human pre-mRNA splicing factor 2 (SF2) to study the relationship between structure and function in RNA processing. Following electrophoresis and digestion in situ with lysyl endoproteinase, the fragments recovered by reverse-phase chromatography were located and sequenced. We are now in a position to determine the phosphorylation sites by similar maps after ^{32}P-phosphate labeling in vivo and in vitro. Interestingly, as we sequenced all of the fragments, we found several peptides derived from an unknown protein that comigrated with SF2 on electrophoresis. Sequence analysis revealed that this protein is another putative RNA splicing factor.

TRANSCRIPTION FACTORS

The phosphorylation of the c-Fos and c-Jun proteins was done in collaboration with T. Curran's group at the Roche Institute (Abate et al., *Oncogene 6:* 2179 [1991]; Baker et al. 1992). Sites on c-Fos and c-Jun proteins were analyzed by phosphorylating in vitro the proteins produced in bacteria as recombinants. Purified proteins as well as truncated versions with various domains deleted were used. Phosphorylation sites on c-Fos occur in regions that have been linked

genetically to the regulation of transcriptional control. Notably, sites for the cell-cycle-regulated protein kinase, $p34^{cdc2}$, were identified, suggesting that there might be significant cell cycle regulation of the transcriptional control by c-Fos. Sites of phosphorylation by $p34^{cdc2}$ were also found on c-Jun protein. One of these sites overlaps a carboxy-terminal site reported to have negative regulatory effects. A second site for $p34^{cdc2}$ was found in the amino-terminal domain close to the region that is different in the v-Jun protein.

RETINOBLASTOMA

The recessive oncogene, retinoblastoma (*Rb*), was analyzed in collaboration with E. Harlow at the Massachusetts General Hospital (Lees et al., *EMBO J. 10:* 4279 [1991]). Rb protein is quite large (105 kD), but it is found in only minute quantities in cells. It is therefore very difficult to identify phosphorylation sites directly from in vivo metabolically labeled proteins. We took a novel approach of using synthetic peptide model substrates to map phosphorylation sites in vitro and then compared these to maps of immunoprecipitated, in-vivo-labeled Rb protein. This approach required that we survey the Rb protein sequence, inferred from the cDNA structure, for potential sites of $p34^{cdc2}$ phosphorylation. We identified clusters of potential sites and synthesized moderately long peptides (30–40 residues) covering these sites. Determination of the stoichiometry and positions of the phosphates was accomplished by a combination of mass spectrometry and protein sequencing. The final compilation of data allowed the assignment of five sites of phosphorylation of Rb by $p34^{cdc2}$ and excluded several sites from phosphorylation in vivo.

Protein Chemistry Core Facility

G. Binns, M. Meneilly, N. Poppito, M. Vandenberg, R. Kobayashi, D.R. Marshak

The Protein Chemistry Core facility provides high technology methods, equipment, and expertise for use by all scientists at Cold Spring Harbor Laboratory. The services provided include protein sequencing, peptide mapping by HPLC, peptide synthesis, HPLC and electrophoretic purification of proteins, mass spectrometry, and amino acid analysis. These methods can be used for structural analysis of the amino acid sequence and composition of a protein, as well as for determining posttranslational modifications. Although peptide synthesis remained approximately constant, amino acid analysis returned to 1990 levels. Protein sequencing and peptide mapping have been aided by the addition of new equipment to the laboratory. We have obtained an Applied Biosystems 473 protein sequencer and have adapted it for either standard Edman or phosphopeptide sequencing. In addition, we have been awarded a large shared instrumentation grant that provides funds for a new HPLC and another protein sequencer. The new HPLC is a Hewlett Packard 1090M, with the new workstation based on DOS operating software. This instrument is networked to our existing HPLC, providing a comprehensive system for chromatography. Now we can do peptide maps and carry out amino acid analysis at the same time.

Structural Analysis of Proteins

R. Kobayashi

Amino acid sequence information of protein has an important role in studying the structure and function of proteins. This year, I introduced a new technique to isolate and separate peptide fragments after digestion of protein with protease while still embedded in polyacrylamide gels. In this way, internal amino acid sequences can be obtained in a minute amount of protein having biological importance. With this new technique, the procedure for obtaining internal amino acid sequences of proteins at a level of 2–10 μg, with a molecular mass of up to 150 kD, became relatively simpler and routine: separate proteins by SDS-PAGE, stain the proteins with Coomassie blue, destain the background of the gel, excise the protein bands, digest the protein in the gel by *Achromobacter* protease I (lysylendopeptidase), extract the peptides, and separate them by reverse-phase high-performance liquid chromatography (RP-HPLC), using an anion exchange pre-column of diethylaminoethyl (DEAE)-derivatized silica. The eluted peptide fragments are then analyzed on an automated protein sequencer. When proteins radioactively labeled with ^{32}P were analyzed, I calculated more than 97% recovery with this technique.

In collaboration with B. Stillman (Cold Spring Harbor Laboratory), I have obtained a partial amino acid sequence of the origin recognition complexes

(ORC; subunits: 50, 53, 56, 62, 65, 72, and 120 kD) and replication factor C (RFC; subunits: 36, 40, 41, and 103 kD) from the yeast *Saccharomyces cerevisiae* and chromatin assembly factor (CAF-1; subunits: 55, 60, and 150 kD) from human. We obtained enough information to isolate these genes for further studies; the sequence analysis is currently under way for human maturation factor (MF- l)

In collaboration with A. Krainer (Cold Spring Harbor Laboratory), I have obtained sufficient sequence information of mRNA splicing factor p52 to isolate its gene. We also made peptide maps to determine the in vivo phosphorylation sites of human mRNA splicing factor 2 (SF2).

Methodological Study of Protein Sequence Analysis

R. Kobayashi

One of my research interests is to develop a new, highly sensitive method for protein sequence analysis. Protein sequence analysis has played a major role in studies on the relationship of protein structure and function. Proteins of biological importance, however, are not easily obtained in sufficient amounts for structure analysis, and this has caused delays in biological discoveries. As the technology for identifying and purifying proteins continues to develop,

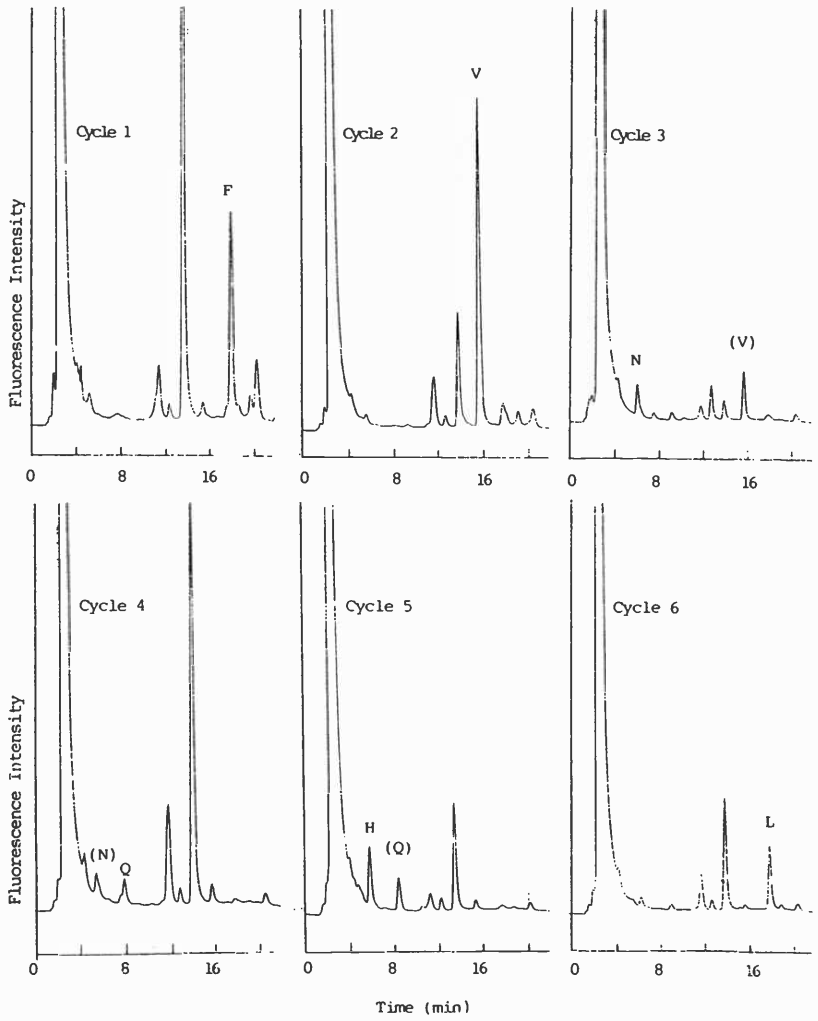

FIGURE 1 Sequence analysis of insulin B-chain (Sequence: FVNQHL----) by the modified Edman degradation.

more sensitive techniques will be required for protein structural analysis. Although the technique that I employed this year allows us to obtain internal sequence information in the amount of a few micrograms of protein, which can only be purified by polyacrylamide gel electrophoresis, we still require more than 1 μg of protein. This amount is still sometimes difficult to obtain for many biologically significant proteins. At this time, there are proteins that are detectable when separated by HPLC after fragmentation of the proteins, but we cannot sequence them. Therefore, I have been trying to improve the sensitivity of protein sequencing by modifying the Edman degradation. This year, I employed aminolytic conversion instead of acid conversion to introduce a fluorescence tag, and I gave up using a new Edman-type reagent for sequencing. I decided to stay with the well-studied phenylisothiocyanate. The Edman chemistry is the same as the original until the extraction step of 2-anilinothiazolinone of amino acids. Here, a fluorescence tag is introduced into these molecules through aminolytic conversion. The sequencing products are detectable by both their fluorescence and their chemiluminescence. To test whether the chemistry worked, 60 pmoles of insulin B-chain was sequenced. The results of the first six cycles from the amino terminus are shown in Figure 1. One fifth of the sequencing products were separated by HPLC and monitored by their fluorescence. Although I did not use very small amounts of starting protein, these results clearly show that it is feasible to use this chemistry to sequence proteins. I am currently synthesizing and purifying all of the standard amino acid derivatives to optimize HPLC separation. Finally, I will apply chemiluminescence detection, which is much more sensitive than fluorescence detection, to the new method in order to study subpicomole levels of peptides.

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PROTEIN SYNTHESIS

M.B. Mathews P. Clarke, S. Green, S. Gunnery, B. Hofmann, C. Kannabiran, M. Kessler, C. Labrie, M.F. Laspia, Y. Ma, L. Manche, G.F. Morris, P. Nahreini, R. Packer, C. Schmedt, D. Taylor, P. Wendel

Research in this laboratory continues to focus on the regulation of gene expression in human cells. We are investigating three systems that function at different levels. The first operates at the level of protein synthesis, where we are studying a protein kinase that controls the rate of translational initiation. The kinase is part of the interferon-induced antiviral defense mechanism and is influenced by viral products that serve to neutralize its action. The second project is concerned with the regulation of a cellular gene at the transcriptional level by a viral oncogene. Expression of the essential DNA replication protein known as proliferating cell nuclear antigen (PCNA) is activated by one of the transforming proteins encoded by adenovirus, as well as by various other stimuli in the normal cell. We are trying to understand how these stimuli impinge on the activity of PCNA in the cell's nucleus. Finally, the AIDS virus HIV-1 (human immunodeficiency virus type 1) exploits a range of regulatory devices, some of them sophisticated and unprecedented. We are interested in controls that govern viral gene expression at both the transcriptional and translational levels, and which may also link viral gene activation to the proliferative status of the cell. During 1992, Piruz Nahreini joined the laboratory as a postdoctoral fellow, Chitra Kannabiran and Mark Kessler moved on to other appointments, and Christian Schmedt worked for several productive months as an exchange student.

Translational Control

P. Clarke, Y. Ma, C. Schmedt, D. Taylor, S. Green, L. Manche, M.B. Mathews

The protein kinase DAI, an acronym for the double-stranded RNA activated inhibitor of translation, is an important element in the host antiviral response that is induced by interferon. The kinase is associated with ribosomes in a wide range of tissues and normally exists in an inactive or latent state. As implied by its name, DAI is specifically activated by double-stranded (ds)RNA, probably of viral origin, but not by single-stranded RNA, DNA, or RNA-DNA hybrids. In some situations, activation of DAI by dsRNA results in a complete inhibition of protein synthesis, whereas under other circumstances, there is selective inhibition of the translation of certain mRNAs. This inhibition of protein synthesis is due to the phosphorylation of the initiation factor eIF-2, which is involved in ternary complex formation during the initiation of translation. The kinase was initially discovered as part of an antiviral pathway, but more recently, it has been implicated in cellular differentiation, inhibition of cellular proliferation, the heat-shock response, and perhaps also the transcriptional induction of gene expression by dsRNA. Our laboratory is endeavoring to understand the molecular mechanism by which DAI is activated and how small RNAs, such as adenovirus VA RNA_I, can block this activation. Recent work has concentrated on identifying the nature of the contacts made between DAI and RNA and on elucidating the processes of enzyme activation and inactivation by these ligands.

To explore the complex interactions between DAI and RNAs, we synthesized the enzyme in vitro and examined its RNA-binding domain. Our findings located the domain at the amino terminus of the protein and revealed that it consists of two copies of a novel RNA-binding motif. The motif is characterized by a high density of basic amino acids, by the presence of conserved amino acid residues, and by a probable α-helical structure (Fig. 1). Deletion of either of the two motifs prevents the binding of RNA, but their relative positions can be exchanged, suggesting that they cooperate to interact with RNA. Mutations within the RNA-binding motifs and duplications of the individual motifs indicated that the first (amino-terminal) copy of the motif plays the more important role. Surprisingly, mutations that impair binding have similar effects on the binding of dsRNAs of various lengths and of adenovirus VA RNA_I, implying that the discrimination between activators and inhibitors takes place subsequent to RNA binding.

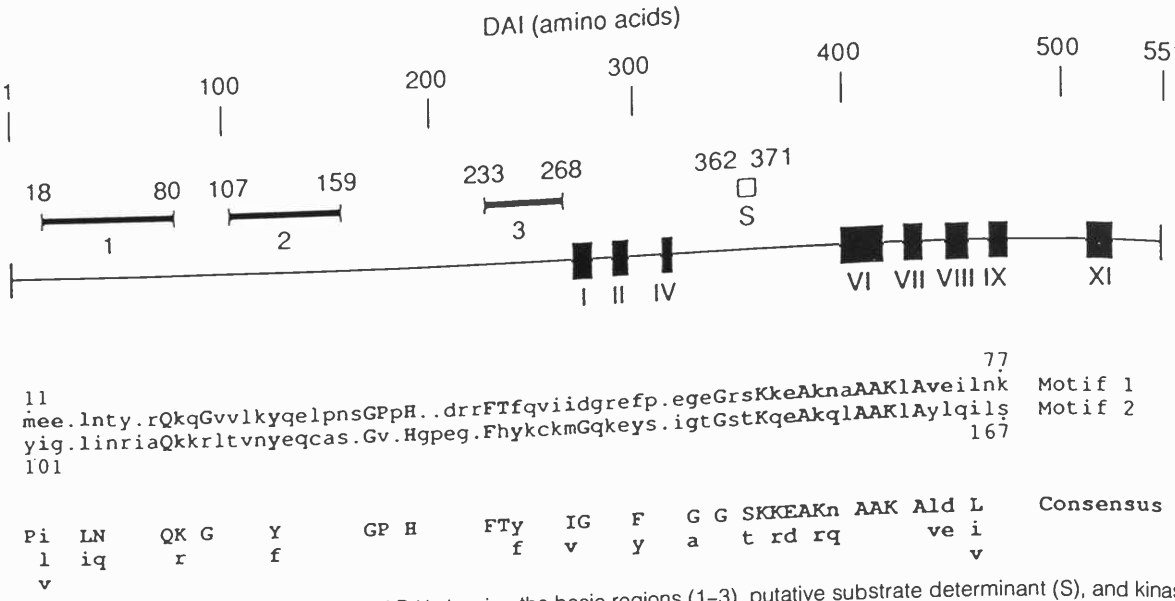

FIGURE 1 Structure of DAI. (*Top*) Map of DAI showing the basic regions (1–3), putative substrate determinant (S), and kinase subdomains (I–XI). (*Bottom*) RNA-binding motifs from DAI and consensus sequence from human and mouse DAI, vaccinia E3L, rotavirus NS34, and TAR RNA-binding protein. Similar or identical amino acids present in the same position in four or more of the sequences are shown in bold type as lowercase or uppercase letters, respectively. Motifs 1 and 2 correspond to basic regions 1 and 2, respectively. The amino-terminal part of each motif is predicted to form an α-helix. (From Green and Mathews 1992.)

On the basis of these results, we produced a truncated form of DAI (p20) that encodes just the first 184 amino acids of DAI, representing the entire RNA-binding domain. This protein was overexpressed in *Escherichia coli* and purified to more than 95% homogeneity. In RNA-binding assays, p20 functions identically to the full-length protein, and gel shift analysis suggests that each copy of the motif interacts with a single turn of the RNA helix. These observations lead us to speculate that kinase activation involves the interaction of the two motifs with suitably spaced helical regions on the activator dsRNA.

DAI is a serine/threonine kinase and its activation as an eIF-2 kinase is correlated with its autophosphorylation. We have begun to identify the location of the autophosphorylation sites and the mechanism of autophosphorylation of DAI. In collaboration with Dan Marshak and Georgia Binns (see Protein Chemistry in this section), the locations of several autophosphorylated sites have been found through peptide mapping and sequencing. These sites may define an auto-regulatory region, separating the enzyme's RNA-binding and kinase domains. Once the phosphorylation sites have been characterized, we will determine which of these phosphorylated serines and threonines are important to the function of the kinase and study the mechanism of autophosphorylation. Preliminary data indicate that some sites on truncated forms of DAI can be phosphorylated intermolecularly, consistent with the view that such a mechanism is used by full-length DAI, but further studies are required to prove the point with the intact protein.

Many viruses have the potential to activate DAI during infection, so it is not surprising that they have evolved mechanisms to inhibit DAI activation and maintain viral replication (sometimes at the expense of host translation). One mechanism involves the accumulation of high concentrations of small, highly structured RNAs that can specifically block the activation of DAI by dsRNA. At late times during adenovirus infection, VA RNA_I plays this role. Earlier work indicated that the function of VA RNA is dependent on its secondary structure and that this consists of a terminal base-paired stem separated from an apical stem-loop by a complex system of stems and loops referred to as the central domain.

To understand this structure in more detail, we have conducted a phylogenetic analysis using six species of VA RNA drawn from three classes of human adenovirus and one simian adenovirus. The VA RNA of the avian adenovirus CELO proved to be too

distantly related to be useful in this study. All the mammalian VA RNAs are built on the same plan, and the comparison served to reveal several interesting features. Although there is little sequence conservation outside regions containing transcription sequences, and the relative proportions of the structural elements also vary, there is a GC-rich sequence in the apical stem that is located in a suitable position to suggest a role in DAI binding. Furthermore, in the 3′ part of the central domain, there is a conserved tetranucleotide (ACCC) that is insensitive to single-strand-specific nucleases but is flanked by two highly sensitive regions and can pair with another conserved tetranucleotide (GGGU) located farther upstream. Support for the view that this structure contributes to the conformation and function of VA RNA comes from mutagenesis of these sequences and from functional assays. Comparing the overall secondary structures of the VA RNAs, it transpires that the most active species have a long apical stem-loop and a trident-structured stem in the central domain. The two moderately active species differ somewhat in the central domain but have a very similar apical stem-loop structure. The VA RNA_{II} forms display only weak translational function and do not appear to possess the same secondary structure in the central domain, presumably accounting for their weak translational function; on the other hand, they are strikingly closely related to one another at the primary sequence level, suggesting that they may play a different role in viral infection.

Previous work indicated that DAI binds primarily to the apical stem of VA RNA_I and that the blocking activity of VA RNA_I resides in the central domain. We have studied the interaction of a large number of VA RNA mutants with DAI in vitro, using a binding assay. The results largely confirm earlier observations: Mutations that disrupt pairing within the apical stem also disrupt binding and function, whereas most mutants within the central domain that lack function nevertheless retain significant binding activity. We have now applied direct approaches to examine the RNA-protein interaction in more detail. Ribonuclease footprinting experiments indicate that DAI protects the base of the apical stem, the complementary conserved tetranucleotides, and other regions in the central domain. The truncated form of DAI, p20, displays somewhat reduced protection of the central domain. Exposure of the complex to chemical probes suggests that the protein does not make sequence-specific interactions with the bases, but more likely interacts with the backbone of the RNA duplex. Moreover, these footprints showed protection of approximately half a turn of the apical stem, in the minor groove of the duplex, consistent with close contacts between the amino terminus of DAI and the backbone of the apical stem. At an altogether different level of resolution, we have collaborated with Luis Jiménez-Garcia and David Spector (Molecular Genetics of Eukaryotic Cells Section) to examine the intracellular distribution of VA RNA and DAI. These two moieties colocalized in the cytoplasm, as anticipated from their function, and both were also found in the nucleus. VA RNA displayed a dotted pattern in the nucleoplasm, consistent with its site of synthesis. Less expectedly, DAI was found in the nucleolus and, to a lesser extent, in the nucleoplasm. Its nucleolar site of distribution might be related to its assembly onto ribosomal particles, but alternative explanations cannot be ruled out at present.

Regulation of PCNA

G.F. Morris, C. Labrie, C. Kannabiran,
R. Packer, M.B. Mathews

Activation of the DNA replication machinery is central to the process of neoplastic transformation. The proliferating cell nuclear antigen, PCNA, plays an essential part in leading-strand DNA replication. It is the auxiliary factor of DNA polymerase δ and functions in leading-strand DNA synthesis during DNA replication. In accord with this function, cellular PCNA levels increase during the oncogenic transformation of rodent cells by the adenovirus E1A gene. The goal of this project is to explore the relationship between transformation and gene activation by elucidating the mechanism of E1A-mediated induction of PCNA gene expression.

The E1A 243R protein (243 residues long), translated from the E1A 12S mRNA, is able to stimulate PCNA transcription and to activate the PCNA promoter in the absence of other viral genes. To define the E1A sequences needed, we assayed 13 different E1A mutants for their ability to *trans*-activate a cotransfected PCNA promoter construct in HeLa cells. Large deletions that in combination span the entire protein severely impair the ability of E1A to induce PCNA expression, whereas smaller deletions and point mutations that target specific cellular protein-binding properties of E1A 243R are less

deleterious to PCNA induction. The results suggest that E1A can activate transcription of the PCNA gene by multiple mechanisms and that, of the known E1A-associated proteins, p300 and p107/cyclin A can mediate the response, whereas p105-Rb and p130 can probably be excluded. However, two E1A mutants that separately bind p300 and p107/cyclin A do not complement each other in the cotransfection assay, implying that the two proteins must be bound to the same E1A molecule or that an as yet unidentified E1A-associated protein is involved.

To define the *cis*-acting sequences involved in the induction of PCNA by E1A 243R, we conducted linker-scanning and site-directed mutagenesis of the promoter. The required promoter region lies between nucleotides −59 and −45 upstream of the transcription initiation site and includes the sequence AGCGTGG immediately upstream of the ATF-binding site previously shown to be important for activation of PCNA by E1A 243R. This PCNA E1A-responsive element (PERE) appears to constitute a complex promoter element as mutation of either the upstream component or the ATF site within the PERE diminishes basal promoter activity and abrogates *trans*-activation by E1A 243R. The PERE is also essential for both basal and E1A-induced expression in the context of the full-length PCNA promoter and is sufficient to confer responsivity to E1A upon a heterologous promoter. Furthermore, the importance of the PERE to PCNA gene expression is highlighted by the occurrence of a similarly positioned homologous sequence in the rat and mouse PCNA promoters and in the promoter for another human DNA replication protein, DNA polymerase β.

We have begun to characterize the proteins that interact with the PERE using biochemical techniques. The PERE is protected from DNase I digestion by nuclear extracts from 293 cells, and gel retardation assays with the PERE have revealed the existence of multiple PERE-associated complexes in 293 cell nuclear extracts. We will now attempt to identify the cellular proteins that compose these complexes. In a complementary genetic approach, we have replaced the PERE with five GAL4 sites and tested the ability of various effectors, expressed as fusions with the GAL4 protein, to mediate activation by E1A. It appears that weak effectors can mediate induction by 243R, whereas E1A reduces activation by strong effectors (Fig. 2). Thus, the PCNA basal promoter is configured in a manner that permits the response of

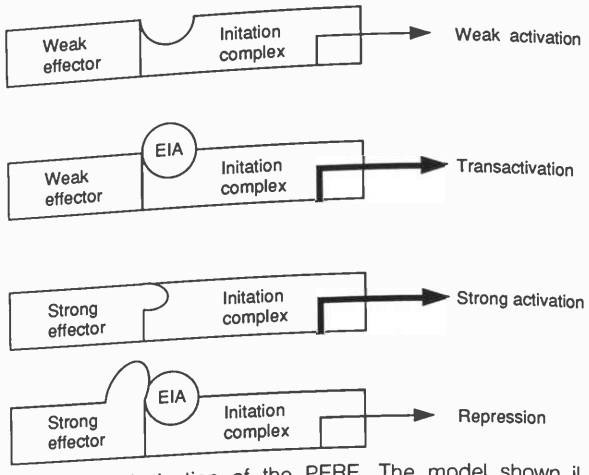

FIGURE 2 Activation of the PERE. The model shown illustrates a possible mechanism for the E1A response of G5PCNA-CAT to effectors of varying strengths. In the absence of effector, a stable initiation complex is not formed on the PCNA promoter and there is no response to E1A. Weak effectors support the stable association of the initiation complex with the promoter, and E1A interacts with the initiation complex in a manner that complements the weak effector. In contrast, strong effectors interact with the initiation complex more productively than the weak effectors. E1A represses transcriptional activation by the strong effectors because it interferes with the interaction of the strong effector with the initiation complex.

transcriptional activators to be modulated by E1A, perhaps by favoring protein-protein interactions that recruit further factors such as TFIID to this TATA-less promoter.

Most of these experiments were conducted in HeLa cells, a transformed cell line derived from a human cervical carcinoma. To extend our analysis of E1A activation of the PCNA promoter to more normal cell types, we employed two kinds of rodent cells, primary baby rat kidney (BRK) cells and cloned rat embryo fibroblasts (CREF). These two cell types display more regulated growth control than HeLa cells and they can be transformed by adenovirus infection. Surprisingly, the PCNA promoter is repressed by E1A in both cell types. As with other promoters repressed by E1A, repression of the PCNA promoter mapped to the p300-binding region of E1A. The *cis*-acting sequences of the PCNA promoter required for repression by E1A reside between nucleotides 234 and 213 upstream of the transcriptional initiation site of the PCNA promoter. Within this sequence is an 18 out of 20

match to a p53-responsive element. Gel mobility shift experiments conducted in collaboration with J. Bischoff (Genetics Section) indicated that this sequence is bound by wild-type, but not mutant, human p53 expressed in yeast. Presumably, the HPV E6-induced loss of p53 function in HeLa cells precludes repression of PCNA-CAT by E1A in these cells. Consistent with our observations in HeLa cells, constructs lacking sequences upstream of –213 are activated by E1A 243R in both CREF and mouse 3T3 cells. Future experiments will address the relationship between E1A repression of PCNA-CAT and p53 function in other cell types.

Regulation of HIV Gene Expression

S. Gunnery, P. Nahreini, M. Kessler, B. Hofmann, M.B. Mathews

Expression of the genes encoded by the human immunodeficiency virus (HIV-1) is critically dependent on a viral protein called Tat which functions in conjunction with a viral RNA sequence known as TAR. These elements cooperate to increase the rate of transcriptional initiation and the efficiency of transcriptional elongation (for a discussion of the mechanisms involved, see below). Previous work, using transient expression assays, indicated that the action of Tat can be influenced by the origin of SV40 DNA replication in such a way as to suggest that transcription from the viral long terminal repeat (LTR) might be linked to replication. Since the activation of HIV transcription is indispensable for the virus to exit from latency and initiate a productive infection, we reasoned that the proximity of the integrated HIV provirus to a DNA replication origin may affect the viral promoter activity and its subsequent pathogenicity. To address this, as well as to extend our previous studies, we are exploiting the ability of recombinant adeno-associated virus (AAV-1) to transfer and stably integrate the reporter gene cassette into human chromosome 19 in a site-specific fashion. In pursuit of this goal, we have recently constructed several chimeric AAV vectors containing the LTR-CAT reporter cassette. In some of these chimeric AAV vectors, a selectable marker gene was inserted to facilitate the isolation of clones containing the integrated HIV gene cassette. Such recombinant AAVs were used to infect HeLa cells, and the process of isolating clones is currently under way. In the near future, the site of LTR-CAT gene integration will be determined using the cloned chromosome-19-specific AAV junction DNA fragment as a probe, and the HIV promoter activity will be monitored in the absence or presence of the Tat protein. We hope that this strategy will provide insights into the effects of host DNA replication on HIV gene expression and perhaps disclose a link between HIV pathobiology and the proximity of the integrated provirus to a cellular replication origin.

The TAR sequence is also capable of influencing HIV gene expression at the translational level. The 5′-untranslated region of all HIV-1 mRNAs contains this sequence, and TAR RNA also exists as a free cytoplasmic form of about 58–66 nucleotides. As reported earlier, this short form of TAR RNA can act like VA RNA to block the activation and autophosphorylation of DAI in a kinase assay in vitro. We have now extended this observation to a cell-free translation system where TAR RNA reverses the inhibition of protein synthesis mediated by dsRNA. TAR RNA folds to form a stem, bulge, and loop, and mutagenic and structural analyses indicate that a stem of at least 14 bp is required for this activity, whereas the TAR loop and bulge required for *trans*-activation by Tat are dispensable. Truncation of the RNA to 68 nucleotides results in the loss of translational rescue ability, suggesting that the short cytoplasmic TAR RNA produced by viral transcription in vivo may not have the capability to suppress activation of the kinase.

To determine the significance of the inhibitory property of TAR RNA in vivo, we employed a transient expression assay. Transfection into 293 cells of a plasmid containing the chloramphenicol acetyltransferase (CAT) gene under the direction of the mouse β-globin promoter leads to activation of DAI and eIF-2 phosphorylation. The resultant poor expression of CAT is greatly stimulated by cotransfection of a plasmid producing the VA RNA which blocks the activation of DAI and consequently improves the expression from the vector. We have now shown that TAR RNA can function in the same way. To express TAR RNA in vivo, we constructed the pVA-TAR plasmid, in which TAR sequences are placed downstream from the strong VA RNA_I gene promoter. Because this RNA polymerase III promoter is intragenic, the transcribed RNA is chimeric: VA-

TAR RNA is 171 nucleotides long, containing the TAR sequence (−3 to +82) flanked by 68 nucleotides of VA RNA sequence on its 5′ side and 9 nucleotides on its 3′ end. The VA RNA sequences that are present in VA-TAR lack both of the features of this RNA that are required for blocking DAI activation, the central domain and the apical stem, and when examined by nuclease sensitivity analysis, the regions derived from VA RNA were unstructured, as evidenced by frequent single-strand cuts. However, the TAR RNA structure in the VA TAR chimera is essentially identical to that in free TAR RNA. Thus, the main structure observed in the molecule was the stem and loop structure of TAR RNA together with a small 3′-terminal stem-loop (Fig. 3C). Cotransfection of pVA-TAR with the reporter plasmid resulted in a stimulation of CAT enzyme activity (Fig. 3A), showing that a longer TAR transcript can stimulate expression in vivo. This finding raises the possibility that the TAR structure at the 5′ end of viral messages exerts its function *in cis*, thereby conferring a translational advantage to HIV mRNA over cellular mRNA.

Transcriptional Regulation by the HIV-1 Tat Protein

P. Wendel, M.F. Laspia

The genome of HIV-1 contains, in addition to the structural genes *gag*, *pol*, and *env*, several novel regulatory genes. One of these, *tat*, is essential and encodes a powerful *trans*-activator that greatly stimulates HIV-1 gene expression. It does so by increasing transcription directed by the promoter located within the long terminal repeat (LTR) of the virus. *Trans*-activation occurs by a novel mechanism in which Tat binds to an RNA element, known as TAR RNA, that is present in the 5′-untranslated region of all HIV mRNAs. In the absence of Tat, the level of HIV-directed transcriptional initiation is low and transcriptional elongation is inefficient, such that the number of transcriptional complexes declines with increasing distance from the promoter (polarity). The binding of Tat to TAR RNA produces dual effects on transcription, increasing initiation of transcription by RNA

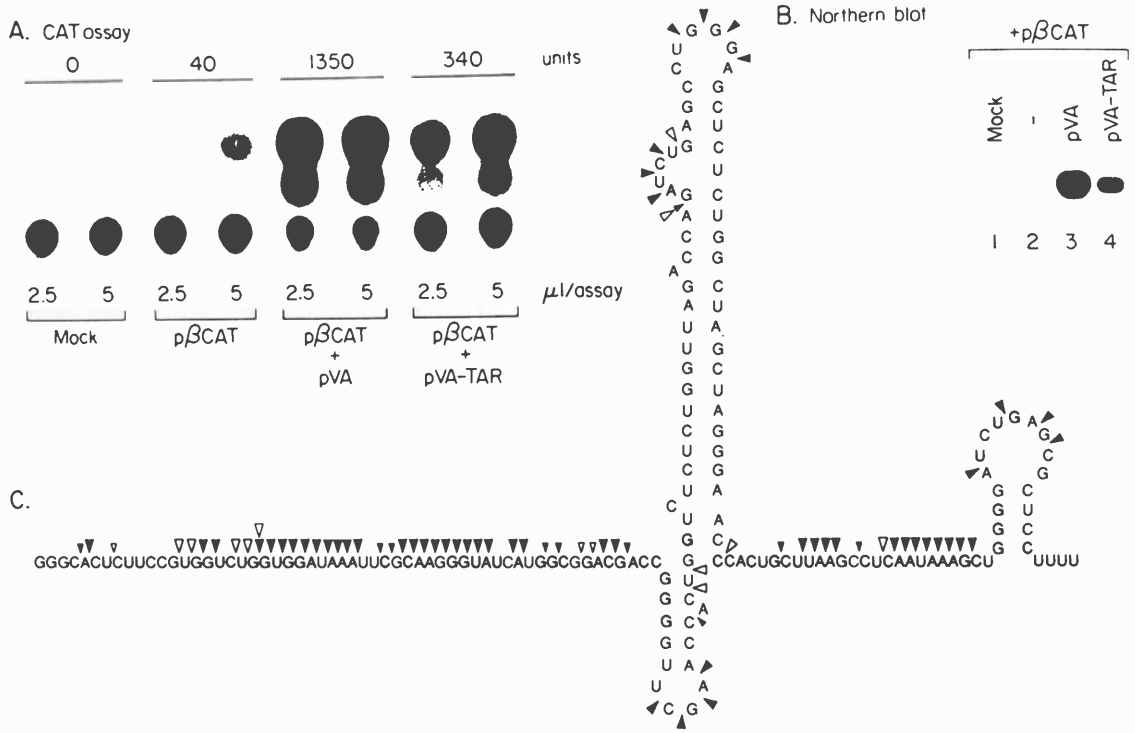

FIGURE 3 Effect of TAR RNA in vivo. (*A*) CAT enzyme activity in lysates of human 293 cells transfected with carrier DNA only (lanes *1,2*), pβCAT plasmid alone (lanes *3,4*), or with pVA (lanes *5,6*), or with pVA-TAR (lanes *7,8*). CAT assays were conducted with two different amounts of lysate. (*B*) Blot of RNA from transfected cells probed with an RNA complementary to the 5′ end of VA RNA. Cells were transfected with salmon sperm DNA only (lane *1*), pβCAT alone (lane *2*), pVA (lane *3*), or pVA-TAR (lane *4*). (*C*) Secondary-structure model for VA-TAR RNA.

polymerase II as well as improving the efficiency of elongation. We are interested in understanding the mechanism by which Tat acts to stimulate HIV transcription, both as a paradigm for regulation of eukaryotic gene expression and because approaches that interfere with *trans*-activation may have therapeutic potential for the treatment of AIDS.

We first began analyzing *trans*-activation by Tat several years ago, in vivo, in a recombinant adenovirus HeLa cell model system. The recombinant adenovirus vector provides an efficient means to deliver an HIV-1 LTR reporter gene fusion into HeLa cells. This enabled us to perform a detailed analysis of the effects of Tat on HIV transcription rates. These studies suggested that Tat did not act as an antiterminator as was thought to be the case at the time. Rather, we found that Tat stimulates transcriptional initiation and generally improves elongation. Furthermore, an examination of the basis of the synergistic stimulation produced by Tat and general activators indicated that stabilization of transcriptional elongation, rather than increased transcriptional initiation, is responsible for synergy. It is thought that synergy between Tat and other viral and cellular activators may contribute to the transition from low levels of gene expression during latency to high-level expression during productive infection.

Since a detailed understanding of the mechanism of Tat action is most likely to emerge from a cell-free approach, we have also analyzed *trans*-activation in vitro. As discussed in last year's Annual Report, characterization of a cell-free system for *trans*-activation indicated that purified, bacterially expressed Tat specifically stimulates HIV-1 transcription in a HeLa cell nuclear extract. An examination of the effects of Tat on promoter proximal and promoter distal transcription has shown that in vitro Tat regulates transcriptional elongation (Fig. 4). The effects of Tat on HIV-directed transcription rates were measured by pulse-labeling transcription and hybridizing the radioactive RNA to contiguous DNA fragments extending across the transcription unit. This provided an estimate of the relative distribution of transcription complexes along the template. In the absence of Tat, the number of transcriptional complexes decreased markedly with increasing distance from the promoter. Although Tat did not significantly increase the number of promoter proximal complexes, it increased the number of promoter distal complexes 15-fold. The magnitude of *trans*-activation by Tat was found to be greatest when examined following an interval of pretranscription during which the efficiency of transcriptional elongation in the absence of Tat decreased. Together, these results suggest that the principal effect of Tat, under these in vitro conditions, is to stabilize elongation. The inability to detect an effect on initiation in vitro may be a consequence of high basal levels of HIV transcription or inefficient reinitiation.

These findings are consistent with Tat acting to preserve the activity of a factor required for efficient elongation or to overcome the action of an inhibitor of elongation. Analysis of the effects of the detergent sarkosyl on HIV transcription has suggested a possible mechanism for how Tat acts to increase HIV-1 transcription. The addition of low concentrations of sarkosyl to transcription reactions increased HIV basal transcription to a level similar to that achieved by Tat alone. Like Tat, sarkosyl stimulated transcription by increasing the efficiency of transcriptional elongation. The combined effects of sarkosyl and Tat on transcription were not additive, implying that they may act on HIV transcription in a similar fashion. Moreover, low basal expression by the HIV promoter may not be due to the assembly of a deficient transcription complex. Rather, stimulation by sarkosyl suggests that HIV transcription may be subject to repression. Since sarkosyl also stimulated transcription from heterologous promoters, repression may be a property shared with other eukaryotic promoters. This hypothesis is supported by the finding that Tat stimulated transcription in vitro from the adenovirus major late promoter with a TAR element cloned downstream from the transcriptional start site. As a working model, we propose that Tat is a gene-specific antirepressor that, when bound to TAR, enables transcriptional complexes to overcome a general inhibitor of elongation.

We have also characterized the function of the HIV-1 TAR element in *trans*-activation by Tat. TAR RNA folds into a structure consisting of a 59-nucleotide paired stem with a trinucleotide bulge and an apical pentanucleotide loop. Tat binding to TAR RNA in vitro is abolished by mutations in the bulge; however, mutations in the apical loop do not reduce the binding of Tat to TAR RNA. On the other hand, mutations that disrupt the stem or change the nucleotide sequence in either the bulge or the apical loop greatly reduce *trans*-activation in vivo. To assess the role of TAR in *trans*-activation, we have examined the ability of synthetic wild type and mutant TAR RNAs to inhibit stimulation by Tat in the cell-free system. Although the addition of 1 μM wild-type TAR RNA, corresponding to nucleotides +1 to +83,

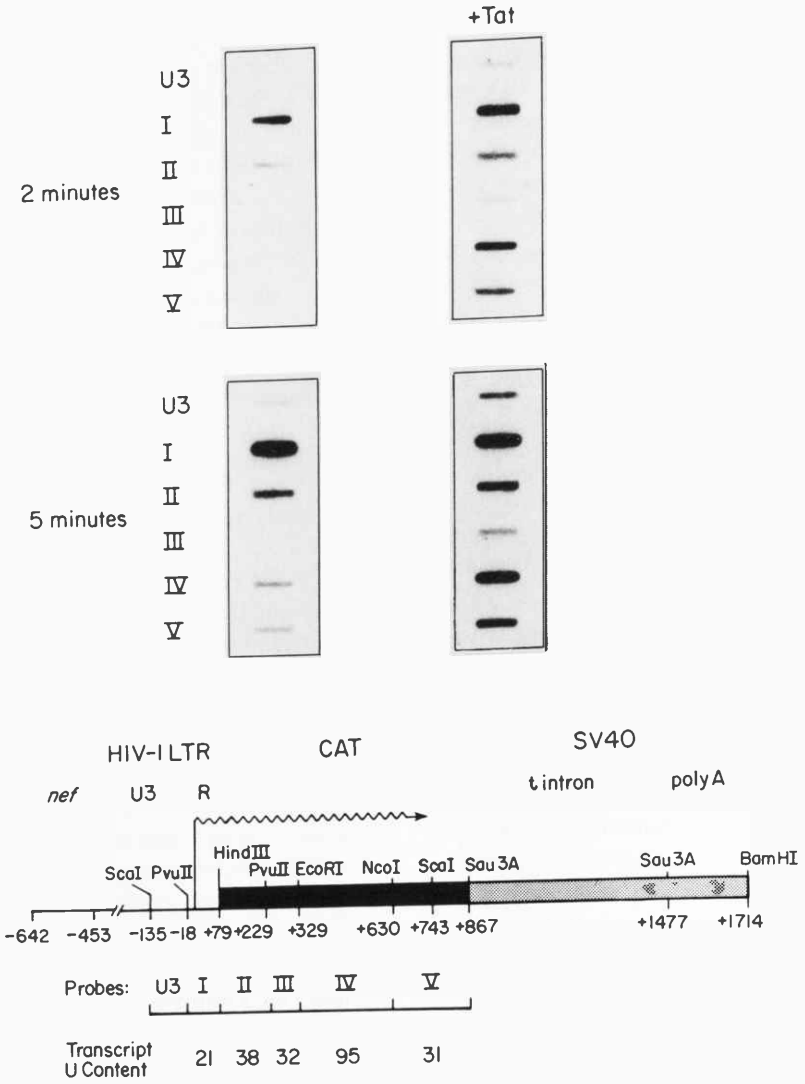

FIGURE 4 Effect of Tat on HIV-promoted transcription rates in vitro. (*Top*) Transcription reactions contained HIV DNA template, 250 ng of Tat, and HeLa cell nuclear extract. Transcription was initiated and, after 30 min, pulse-labeled with [α-^{32}P]UTP for 2 min or 5 min as indicated. RNA was isolated and hybridized to filter-bound, antisense DNA probes corresponding to portions of the HIV-directed transcription unit. Following washing, filters were subjected to autoradiography. (*Bottom*) Schematic of the HIV-1 DNA template (*top line*), the DNA probes used to analyze HIV transcription rates (*second line*), and the uridine content of each transcript (*third line*). The amount of radioactivity, normalized for uridine content, hybridizing to each probe is an estimate of the relative number of transcription complexes located in that region of the gene.

strongly inhibited *trans*-activation by Tat, the addition of mutant TAR RNA containing a deletion of nucleotides +35 to +38 failed to compete *trans*-activation when added at 8 μM. In addition, synthetic TAR RNA with mutations in either the bulge or the apical loop also failed to inhibit *trans*-activation. These results indicate that the integrity of the stem as well as the sequence of both the trinucleotide bulge and apical loop is essential for TAR RNA to inhibit *trans*-activation by Tat. This suggests that Tat may bind cooperatively with a cellular protein that has specificity for the apical loop. We are currently determining whether inhibition of Tat responsiveness by synthetic TAR RNA is due to competition for the

binding of Tat or the binding of a cellular protein(s) that increases the affinity of Tat for TAR.

At the present time, little is known about cellular factors that might be involved in *trans*-activation. However, genetic and biochemical evidence supports the idea that cellular cofactors, either activators and/or repressors, are required for Tat responsiveness. As mentioned above, Tat appears to bind cooperatively with a cellular cofactor(s) to TAR RNA. We are attempting to purify and characterize this cellular factor(s) from fractionated nuclear extracts using the ability to overcome inhibition of *trans*-activation in vitro by competitor TAR RNA or the ability to bind to TAR RNA as assays. An additional strategy that is being utilized to purify a cellular cofactor(s) required for Tat responsiveness is to obtain a "minimal system," through the purification of transcription factors, that supports basal transcription but not Tat-stimulated transcription. At least eight cellular activities are required for basal transcription of mRNA promoters: TFIIA, IIB, IID, IIE, IIF, IIH, IIJ, and RNA polymerase II. We are determining whether these factors are necessary and sufficient for a Tat response in a reconstituted system with purified factors. Initial studies indicate that a fractionated system consisting of nuclear extracts that have been fractionated by chromatography on P11 phosphocellulose is capable of supporting basal transcription but not *trans*-activation by Tat. We are utilizing this system to attempt to purify a cellular protein(s) required for Tat responsiveness by complementation of activity.

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RNA SPLICING

A.R. Krainer E. Birney, J. Cáceres, B. Dong, A. Hanamura, D. Horowitz, G. Joshi-Tope, D. Kozak, A. Mayeda, V. Chua, K. Otto, M. Wallace

MECHANISMS OF MAMMALIAN PRE-mRNA SPLICING AND SPLICE SITE SELECTION

RNA splicing is a required step in the expression of most eukaryotic protein-coding genes. Alternative pre-mRNA splicing is a widespread mechanism for generating structurally and functionally distinct protein isoforms from single genes, often in a tissue-specific, developmentally regulated, or physiological-

ly controlled manner. Both types of mechanisms involve multiple protein components as well as RNA components that are part of small nuclear ribonucleoprotein (snRNP) particles. RNA-RNA base-pairing interactions among snRNAs and pre-mRNA are important determinants of splice site recognition and in addition probably participate in forming the active sites for the *trans*-esterification reactions within the spliceosome. The role of spliceosomal proteins is less well understood, although protein-RNA and protein-protein interactions are also essential for pre-mRNA splicing. Several required protein-splicing factors are thought to play important roles in spliceosome assembly, splice site selection, and conformational rearrangements coupled to ATP hydrolysis. Other possible roles include regulation of active site accessibility, turnover of snRNPs, and positive and negative regulation of alternative splice site selection, for example, through posttranslational modification. Some protein factors might also contribute chemical groups to the *trans*-esterification active sites. Our laboratory has focused on the identification, purification, and molecular characterization of protein factors that are necessary for the catalysis of splicing and/or for the regulation of alternative splice site selection in vitro.

STRUCTURE, FUNCTION, AND EXPRESSION OF HUMAN SPLICING FACTORS SF2/ASF AND hnRNP A1

We showed previously that human pre-mRNA splicing factor SF2/ASF has an activity required for splicing in vitro and promotes utilization of proximal alternative 5′ splice sites in a concentration-dependent manner by opposing heterogeneous nuclear RNP (hnRNP) A1. J. Cáceres introduced selected mutations in the amino-terminal RNA recognition motif (RRM) and the carboxy-terminal arginine/serine (RS) domain of SF2/ASF and assayed the resulting recombinant proteins for constitutive and alternative splicing in vitro and for binding to pre-mRNA and mRNA. Mutant proteins inactive in constitutive splicing continued to affect alternative splice site selection, demonstrating that these activities involve distinct molecular interactions. Specific protein-RNA contacts mediated by solvent-exposed Phe-56 and Phe-58 in the RNP-1 submotif of the SF2/ASF RRM are essential for constitutive splicing, although they are not required for RRM-mediated binding to pre-mRNA. The RS domain is also required for efficient constitutive splicing activity, and both arginine and serine residues are important for biochemical activity. Analysis of domain deletion mutants showed that the RRM exhibits strong RNA-binding cooperativity with a central degenerate RRM repeat and that these two domains are sufficient for alternative splicing activity in the absence of an RS domain.

A related mutagenic analysis of hnRNP A1 structure and function is being carried out by A. Mayeda in collaboration with Steve Munroe (Marquette University). The ability of hnRNP A1 to promote use of certain distal alternative 5′ splice sites appears to require specific protein-RNA contacts mediated by pairs of conserved phenylalanine residues (analogous to those of SF2/ASF) in the RNP-1 submotifs of each of the two RRMs, although these residues are not required for general RNA binding. The glycine-rich carboxyl terminus of hnRNP A1 is also essential for alternative splicing activity. These mutants are currently being analyzed for their effects on the general splicing stimulatory activity of A1 and on its RNA annealing activity. Previous work on hnRNP A1 has shown that it binds single-stranded nucleic acids in a sequence-independent fashion and in a cooperative manner due to protein-protein interactions between carboxy-terminal domains. However, in crude extracts, distinct binding preferences for 3′ splice sites, as well as changes in the ability to cross-link this protein to pre-mRNA depending on the integrity of snRNAs, have been shown in other laboratories.

We have proposed that the intracellular ratios of SF2/ASF and hnRNP A1 may control the specificity of splice site selection and that in vivo regulation of one or both of these activities may play an important role in the tissue-specific or developmental regulation of alternative splicing. A. Hanamura measured the levels of SF2/ASF and hnRNP A1 polypeptides during adenovirus and SV40 productive infections by immunoblotting with a monoclonal anti-SF2/ASF antibody (generated by D. Kozak with C. Bautista and M. Falkowski, of the CSHL Monoclonal Antibody Facility) and a monoclonal anti-hnRNP A1 antibody (provided by G. Dreyfuss). Only minimal variation in the ratio of SF2/ASF and hnRNP A1 was observed, and altered mobilities that might reflect differences in posttranslational modifications were not seen. It remains possible that these viruses control the alternative splicing patterns of some of their transcripts by altering the activities, rather than the total concentration, of SF2/ASF and/or hnRNP A1 through interaction with other components. For example, J. Stévenin and colleagues proposed that long

adenovirus transcripts sequester SF2/ASF or similar factors, leading to a lower effective concentration, which results in a shift toward late splicing patterns. We previously showed that increasing the concentration of hnRNP A1 relative to that of SF2/ASF leads to activation of the late E1A 9S 5′ splice site and repression of the 13S and 12S 5′ splice sites in vitro.

A. Hanamura has documented substantial variation in the natural ratio of SF2/ASF to hnRNP A1 in different rat tissues. In addition, significant changes were also observed among different cell lines, for example, in response to transformation (in collaboration with J. Cáceres and with B.R. Franza), which might explain some of the alterations of alternative splicing patterns that are commonly seen in transformed cells. These studies of variations in the relative levels or activities of SF2/ASF and hnRNP A1 have to take into account the existence of complex gene families, as well as isoforms generated by alternative splicing and posttranslational modification. Interestingly, a previously documented but unknown posttranslational modification of hnRNP A1, termed A1x, appears to eliminate alternative splicing activity. We have also shown that SF2/ASF has multiple phosphates, but we do not know at present the consequence of phosphorylation on activity. In collaboration with R. Kobayashi, G. Binns, and D. Marshak (see Protein Chemistry, this section), A. Hanamura and A. Mayeda are attempting to map the posttranslational modification sites in SF2/ASF and in hnRNP A1x. Mapping and identification of these modifications should allow further studies of their potentially regulatory roles.

THE SR FAMILY OF NUCLEAR PHOSPHOPROTEINS

A conserved family of nuclear phosphoproteins was recently identified in the laboratory of M. Roth (Fred Hutchinson Cancer Research Center, Seattle). In collaboration with the Roth laboratory, A. Mayeda showed that SF2/ASF is one member of this SR family and that a different member of the same family from *Drosophila* could substitute for SF2/ASF in general or alternative splicing assays with HeLa extracts. Other members of the SR family have several conserved structural features and similar functional properties. Another example is the human splicing factor SC35, which shares 31% homology with SF2/ASF. In collaboration with X.-D. Fu and T. Maniatis (Harvard University), A. Mayeda found that either protein can reconstitute the splicing activity of S100 extracts and of SC35 immunodepleted nuclear extracts. We found that SC35, like SF2/ASF, can affect alternative 5′ splice site selection by antagonizing hnRNP A1 (Fig. 1). In addition, we found that both SF2/ASF and SC35 also favor the proximal site in pre-mRNA-containing duplicated 3′ splice sites but that this effect is not modulated by hnRNP A1. Thus, SF2/ASF and SC35 appear to have essentially equivalent splicing activities in vitro.

The SR family members so far cloned in several laboratories include human SF2/ASF, human SC35, *Drosophila* SRp55 and its closely related variant B52, mouse X16 (identical to human SRp20),

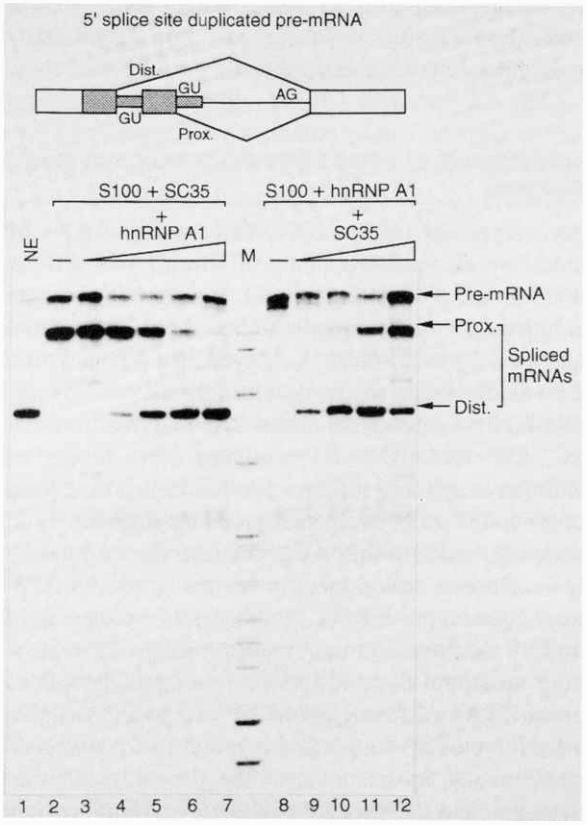

FIGURE 1 SC35 and hnRNP A1 affect alternative 5′ splice site selection in opposite ways. S100 extract (6 μl) was complemented with 3 μl of human SC35 expressed in baculovirus (lanes *2–7*) and increasing amounts of human hnRNP A1 expressed in *E. coli* (lane *2*, no addition; lane *3*, 0.5 μl; lane *4*, 1 μl; lane *5*, 2 μl; lane *6*, 4 μl; lane *7*, 6 μl) or with 3 μl of hnRNP A1 (lanes *8–12*) and increasing amounts of SC35 (lane *8*, no addition; lane *9*, 0.75 μl; lane *10*, 1.5 μl; lane *11*, 3 μl; lane *12*, 6 μl). Nuclear extract (lane *1*, 8 μl) was included as a control. The substrate is a model β-globin pre-mRNA with duplicated 5′ splice sites. The positions of the mRNA products generated by selection of proximal or distal 5′ splice sites are indicated.

Drosophila rbp1, and an SR protein from *Arabidopsis*. Fragmentary protein sequence has been reported for several other mammalian SR proteins, and a closely related cDNA fragment is available from *Caenorhabditis elegans*. In addition to the extensive primary structure similarity between different SR family members from the same or from different species, actual homologs are extremely conserved through evolution. Thus, for example, whereas human SF2/ASF and human SC35 share 31% amino acid sequence identity, human and mouse SRp20 and human and mouse SF2/ASF are 100% identical. Therefore, although individual SR proteins appear to be functionally equivalent or very similar in the in vitro assays currently available, it seems certain that individual family members are not functionally redundant, but rather have specific properties in vivo.

ALTERNATIVE 3′ SPLICE SITE SELECTION AND EXON SKIPPING

As mentioned above, SF2/ASF and hnRNP A1 modulate alternative splicing in vitro of pre-mRNAs that contain 5′ splice sites of comparable strength competing for a common 3′ splice site. Using natural and model pre-mRNAs, A. Mayeda (in collaboration with D. Helfman, see Molecular Genetics of Eukaryotic Cells Section) examined whether the ratio of SF2/ASF to hnRNP A1 regulates other modes of alternative splicing in vitro. We found that an excess of SF2/ASF effectively prevents inappropriate exon skipping, and also influences the selection of mutually exclusive tissue-specific exons in natural β-tropomyosin pre-mRNA. In contrast, an excess of hnRNP A1 does not cause inappropriate exon skipping in natural constitutively or alternatively spliced pre-mRNAs. Although hnRNP A1 can promote alternative exon skipping, this effect is not universal and depends, for example, on the size of the alternative exon and the strength of the polypyrimidine tract in the preceding intron (Fig. 2). This was shown with model substrates, kindly provided by R. Kole (University of North Carolina, Chapel Hill). With appropriate alternative exons, an excess of SF2/ASF promotes exon inclusion, whereas an excess of hnRNP A1 causes exon skipping. We proposed that constitutive splice sites have evolved in conjunction with appropriate sequence and structural contexts to match exclusively appropriate pairs of 5′ and 3′ splice sites so as to avoid exon skipping, regardless of variations in the levels of SF2/ASF, hnRNP A1, and similar regulatory factors. In contrast, alternative splice sites that are in *cis*-competition have more balanced relative strengths and are therefore susceptible to variations in the levels of such factors.

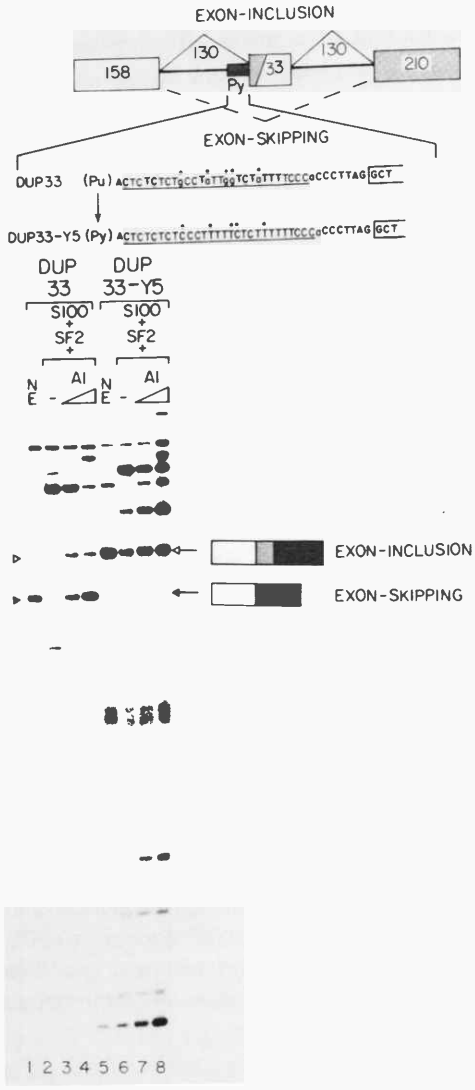

FIGURE 2 Effect of upstream polypyrimidine tract composition on hnRNP A1-promoted exon skipping. The structures of the model pre-mRNAs and the sequences of the polypyrimidine tracts and 3′ splice sites in each upstream intron are shown. The five dots indicate the nucleotide differences (transversions) between the two substrates. The exon and intron sizes are indicated in nucleotides. The structures of the two mRNAs resulting from alternative exon inclusion or skipping are represented on the right. The reactions contained nuclear extract (NE, 6 μl), or S100 extract (6 μl) plus SF2/ASF (0.7 μl) plus recombinant hnRNP A1 (lanes *2* and *6*, none; lanes *3* and *7*, 1 μl; lanes *4* and *8*, 4 μl).

Using model substrates containing alternative 3′ splice sites, A. Mayeda has identified and partially purified activities that stimulate proximal (SF6) or distal (SF7) 3′ splice sites. SF6 activity turned out to cofractionate with SR proteins, and, for example, either SF2/ASF or SC35 can stimulate proximal 3′ splice sites when present in relatively high concentrations. SF7 appears to be distinct from SR proteins and from hnRNP A1, and its purification is almost complete. This factor will be used together with SF2/ASF and hnRNP A1 to investigate the molecular mechanisms of splice site selection in vitro.

OTHER ESSENTIAL PROTEIN SPLICING FACTORS

We are continuing to purify and characterize additional protein factors that are essential for one or both RNA cleavage-ligation reactions. Using selective inactivation and biochemical complementation strategies, G. Joshi-Tope (now at Brookhaven National Laboratory) and D. Horowitz have identified two novel activities required for cleavage at the 5′ splice *site and lariat formation. These activities have been chromatographically separated, and their purification is in progress. B. Dong used immunological and genetic approaches to attempt to identify and isolate cDNA homologs of the *Saccharomyces cerevisiae* PRP18 splicing factor, which is required for 3′ splice site cleavage and exon ligation (in collaboration with D. Horowitz and with J. Abelson, California Institute of Technology). Recently, B. Dong purified a 54-kD HeLa cell polypeptide that reacts strongly with affinity-purified anti-PRP18 polyclonal antibodies. In collaboration with R. Kobayashi (see Protein Chemistry, this section), partial peptide sequence of p54 was obtained and used to design degenerate primers for reverse transcriptase–polymerase chain reaction (RT-PCR). The resulting unique sequence DNA probe was used to isolate full-length HeLa cDNAs encoding p54. Although p54 turned out only to share a fortuitous epitope with yeast PRP18, its amino acid sequence reveals the presence of two RRMs and significant sequence similarity to a number of RNA-binding proteins and mammalian splicing factors. Immunological and biochemical approaches will be used to study the RNA-binding properties of p54 and its possible involvement in pre-mRNA splicing. The fortuitous cross-reactivity of p54 with PRP18 may explain our previous immunocytochemical and biochemical data with anti-PRP18 antibodies and human cells. Therefore, the existence of a true PRP18 homolog in human cells remains uncertain.

CONSERVED MOTIFS IN PROTEIN SPLICING FACTORS

E. Birney (now at Balliol College, Oxford) and S. Kumar (in R.J. Roberts' laboratory, now at New England Bio-Labs) have carried out a detailed sequence analysis of conserved motifs in metazoan protein-splicing factors and related RNA-binding proteins. This analysis, still in progress, includes the characterization of RRMs and the construction of a complete RRM database, as well as statistical analysis of arginine-serine repeats and other simple repeats found in RNA-binding proteins. Several proteins in the databases were found to contain previously unnoticed RRMs and homologies with known splicing factors, which strongly suggest that they are RNA-binding proteins. One example of this analysis is the finding of significant homology between an open reading frame in the recently sequenced *S. cerevisiae* chromosome III and the large subunit of the human splicing factor U2AF (Fig. 3). Both proteins contain a short amino-terminal RS domain followed by three RRMs, the last of which is atypical. If the YCL11c open reading frame is a functional homolog of $U2AF^{65}$, the similarities in the splicing machinery of yeast and metazoans would extend to non-snRNP factors, in addition to the known similarities in snRNAs and snRNP polypeptides. It is of interest that U2AF recognizes the conserved polypyrimidine tract of metazoan 3′ splice sites and that such extensive tracts are generally absent from *S. cerevisiae* 3′ splice sites. In addition, the above sequence represents the first example, to our knowledge, of an RS domain, albeit a short one, in a yeast protein.

The RS domains of $Su(w^a)$ and tra have been shown to be required for targeting of these proteins to the nucleoplasmic speckle region, where splicing factors reside. The RS domains of human $U2AF^{65}$ and SF2/ASF are required for constitutive splicing in vitro. It is unclear at present what constitutes a minimal RS domain, both from statistical relevance and from protein structural standpoints. Sequence analysis of RS domains showed that very few proteins in the databases contain significant repeats of interspersed arginine and serine residues (Fig. 4). Virtually all of these proteins have been directly or indirectly implicated in constitutive or regulated splicing. Many of these proteins also contain one or more RRMs. The proteins of the SR family have character-

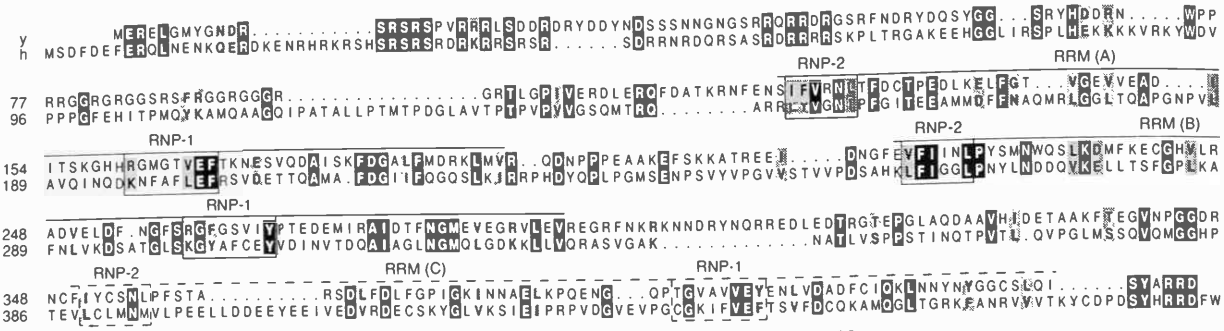

FIGURE 3 Similarity in sequence and domain organization between human U2AF^{65} and an open reading frame from chromosome III of *S. cerevisiae*. The alignment of the YCL11c ORF protein (*top* line) and U2AF^{65} (*bottom* line) was generated with the GCG program Bestfit, with a gap weight 3.5 and gap extension 0.1. Black boxes represent identical matches; stippled boxes represent conservative changes (V=L=I, F=Y=W, R=K, Q=N, D=E, S=T). RRMs are shown by horizontal lines; RNP-1 and RNP-2 submotifs are boxed. The broken line for the carboxy-terminal RRM indicates a weak match to the consensus, particularly for RNP-2 of hU2AF^{65}

istic, highly clustered repeats of consecutive RS or SR dipeptides at their carboxyl termini, an amino-terminal RRM, and in some cases a central degenerate RRM, in addition to other distinctive features. In other proteins with RS domains, the arginine and serine residues are more dispersed and show less periodicity.

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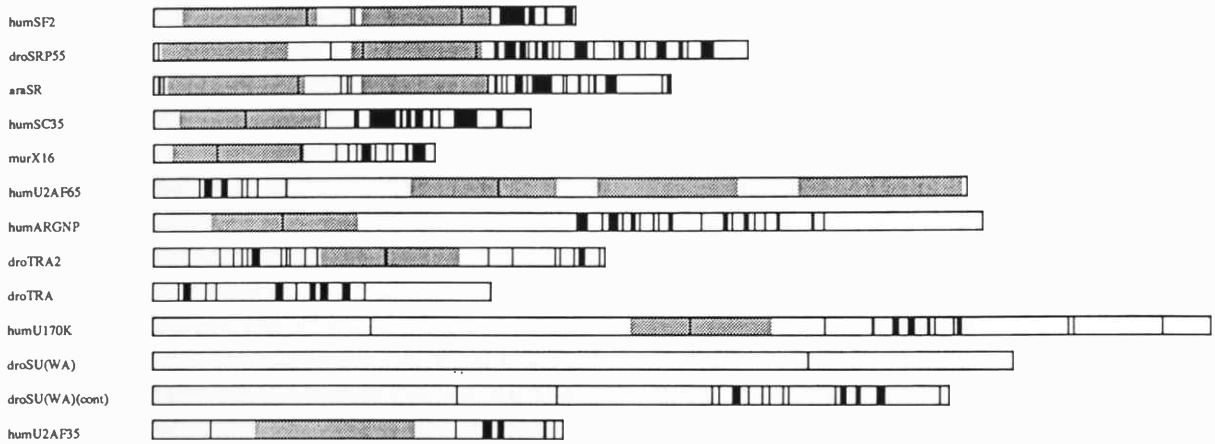

FIGURE 4 Distribution of arginine-serine dipeptides in proteins with RS domains. The boxes represent the indicated proteins, drawn to scale. Prototype or degenerate RRMs are indicated with gray shading. Each arginine or serine residue within an RS or SR dipeptide is indicated by a thin vertical black line. The proteins of the SR family include human SF2/ASF, human SC35, *Drosophila* SRp55, mouse X16, and a protein from *Arabidopsis*. Other species homologs are not shown. Other proteins with RS domains shown are the 65- and 35-kD subunits of human U2AF, an arginine-rich nuclear protein of unknown function, the 70K polypeptide of U1 snRNP, and the *Drosophila* splicing regulators transformer, transformer2, and suppressor-of-white-apricot. The E2 proteins of some but not all human papillomavirus isolates also contain an RS domain, not shown here. The degenerate RRMs of the human arginine-rich protein and of U2AF^{35} were not reported previously.

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TRANSCRIPTIONAL REGULATION

W. Herr
M. Tanaka

R. Aurora, M.Cleary, G. Das, R. Freiman, G. Henry, C. Hinkley, J.-S. Lai, V. Meschan, W. Phares, J. Reader, S. Stern, W. Tansey, W. Thomann, K. Visvananthan, A. Wilson

We use viruses to probe the mechanisms of transcriptional regulation in mammalian cells. Viruses provide simple regulatory systems in which the cellular transcriptional apparatus is frequently usurped to regulate viral gene expression in a temporal cascade. The cellular changes undergone during this process can be more easily compared than during cellular differentiation because initiation of the process by infection can be rapid and discrete.

During the last decade, we have used three primate viruses to study transcriptional regulation. Our earlier studies concentrated on the structure of transcriptional enhancers, which have the ability to activate transcription even when positioned at a large distance from the transcriptional start site. Our genetic studies with the simian DNA tumor virus SV40 showed that the SV40 enhancer is composed of regulatory modules that can cooperate with one another or duplicates of themselves to stimulate transcription. Analysis of the proteins that bind to these regulatory modules led to the purification of the octamer motif (ATGC AAAT)-binding protein Oct-1. This is a cellular protein that is expressed in a broad array of cells and is a close relative of another octamer motif-binding protein called Oct-2. Unlike Oct-1, Oct-2 is more restricted in its expression pattern, being expressed in B cells where it is believed to be involved in activation of immunoglobulin promoters. Both Oct-1 and Oct-2 are members of the POU family of homeodomain proteins. POU proteins are distinguished from other homeodomain proteins because they contain two helix-turn-helix type DNA-binding domains, a homeodomain and POU-specific domain, fused together in the form of a bipartite DNA-binding domain.

Although Oct-1 and Oct-2 recognize the same transcriptional regulatory motif, they preferentially activate different types of promoters: Oct-1 is better able to activate transcription of the U2 small nuclear RNA gene, a ubiquitously expressed gene involved in RNA splicing, and Oct-2 is better able to activate a typical mRNA promoter. This differential promoter activation is achieved by the use of promoter-selective transcriptional activation domains that, even when attached to the same DNA-binding domains, are able to activate different types of promoters. This

differential activation probably results from selective interactions with differing promoter-proximal initiation complexes.

Another mechanism used by Oct-1 and Oct-2 to elicit different programs of gene expression is through differential interaction with the herpes simplex virus (HSV) protein VP16. VP16, also referred to as Vmw65 and α-TIF, is an HSV virion protein that upon infection is released into the cell and forms a multiprotein complex with Oct-1 and a second cellular protein called HCF (or C1, VCAF, or CFF by other investigators), on a class of *cis*-regulatory elements called TAATGARAT (R = purine). VP16 first binds to HCF, which primes VP16 for association with Oct-1 in a DNA-dependent complex. VP16 can associate with Oct-1, but not effectively with Oct-2, by recognizing the Oct-1 homeodomain, which differs from the Oct-2 homeodomain at seven positions that are exposed when Oct-1 and Oct-2 are bound to DNA. Association with VP16 can result in two important changes in the activity of Oct-1: First, VP16 contains a very potent acidic mRNA-promoter activation domain that alters the transcriptional activation properties of Oct-1, and second, VP16 can recruit Oct-1, but not Oct-2, to a new *cis*-regulatory site, thus essentially changing the DNA-binding specificity of one but not the other of two proteins that otherwise display the same DNA-binding specificity. It is in this manner that we have been using HSV to probe the mechanisms of transcriptional regulation in mammalian cells.

We use human immunodeficiency virus type 1 (HIV-1) to study promoter variation in infected individuals. HIV provides an excellent model to study such variation, because, like other retroviruses, it mutates rapidly and because unfortunately there are many known infected individuals from whom variants can be isolated. Below are descriptions of accomplishments we have made during the past year.

The Structure of the Oct-1 POU-specific Domain Is Strikingly Similar to That of the Bacteriophage λ Repressor DNA-binding Domain

R. Aurora [in collaboration with N. Assa-Munt, R. Mortishire-Smith, and P. Wright, The Scripps Research Institute]

We have determined the solution structure of the Oct-1 POU-specific (POU_S) domain by NMR. Figure 1 shows a model of the deduced structure. The structure is remarkably similar to the bacteriophage λ and 434 repressor DNA-binding domains. These DNA-binding domains contain the helix-turn-helix (HTH) motif. The HTH motif is found in a variety of DNA-binding proteins but is not itself an independently stable domain; other structural components are required to form a stable structure. This stabilization can be achieved in a number of ways. For example, in the tri-α-helical homeodomain, a single helix closes off the hydrophobic face of the HTH structure, whereas in the bacteriophage λ and 434 repressors, two helices, the first and fourth, serve the same purpose.

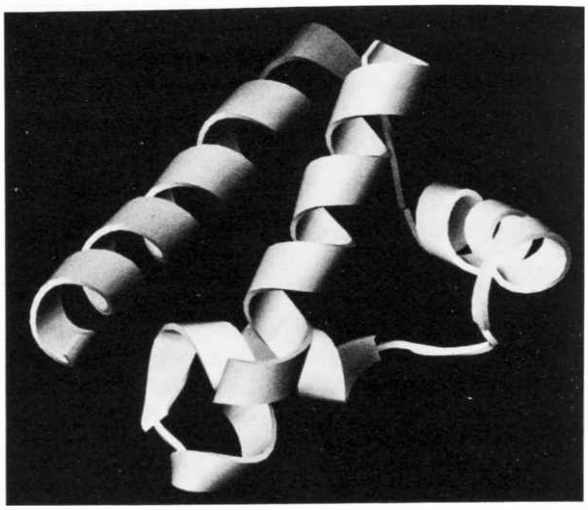

FIGURE 1 Model of the Oct-1 POU-specific domain. The Oct-1 POU-specific domain is shown with ribbons indicating α helices. The amino terminus is at the bottom left and the carboxyl terminus is at the upper right. In this view, helix 1 is at the left, helix 2 is at the right, helix 3 extends horizontally from right to left at the back, and helix 4 is in the center foreground. Helix 3 is the putative DNA recognition helix and helix 2 is where the human dwarfism mutation in the Pit-1 POU-specific domain lies.

The Oct-1 POU_S domain has the same tetra-α-helical HTH-containing structure as the bacteriophage λ and 434 repressors. The POU_S structure differs, however, because it contains an unusual HTH structure, in which the first helix and the turn of the HTH structure are extended. This extended structure means that sequence similarity between the POU_S and bacteriophage repressor DNA-binding domains is not readily detectable. This result explains why the link between the POU_S domain and the two bacteriophage repressor DNA-binding domains was not

made prior to elucidation of the POU_S domain structure.

The POU_S domain structure, together with the known effects of a POU_S domain mutation, suggests a further similarity between the human POU domain proteins and bacteriophage repressors. A human dwarfism mutation in the pituitary POU domain protein Pit-1 affects the ability of this protein to activate transcription but has little effect on DNA binding; presumably, this mutation affects protein-protein interactions important for positive control. This missense mutation is located within the first of the two HTH helices, the same region in which mutations with similar effects lie in the λ repressor. Thus, apparently similar defects in a human and bacteriophage transcriptional activator can cause changes in either human or λ bacteriophage development.

The Oct-1-encoding mRNA 5′ Leader Does Not Contain an Expected AUG Initiation Codon

R. Freiman

Of the many Oct-1-encoding cDNAs we have isolated previously, none extended far enough to contain the likely translational initiation codon. To extend the 5′ cDNA sequences further, we have employed the polymerase chain reaction (PCR) procedure. By this strategy, the *oct*-1 mRNA sequences were extended by 625 nucleotides. Curiously, although the Oct-1-encoding open reading frame extends for 79 codons upstream of the previously described AUG initiation codon, the extended reading frame does not contain any other prototypical AUG initiation codon. There is, however, an unusually large and stable RNA secondary structure. We are currently testing whether translation of Oct-1 initiates at a non-AUG codon.

Enhanced Activation of the Human Histone H2B Promoter by the Oct-1 Variant Oct-1B

G. Das

We have previously described an *oct*-1 cDNA resulting from alternative splicing that has the potential to encode a carboxy-terminally truncated form of Oct-1. This variant form is called Oct-1B and carries a unique carboxy-terminal 12-amino-acid extension. This 12-amino-acid extension is almost entirely composed of glutamine, proline, and threonine residues; such residues are typical of mRNA promoter activation domains. Previously, we have found that Oct-1 does not activate mRNA promoters effectively. Because of the Oct-1B carboxy-terminal structure, however, we tested the ability of Oct-1B to activate the histone H2B promoter. We found that indeed, in a transient cotransfection assay, Oct-1B activates the histone H2B promoter better than does Oct-1. In fact, Oct-1B is as effective an activator of histone H2B transcription as the mRNA-promoter activator Oct-2.

Structure of the VP16-associated Protein HCF

A. Wilson [in collaboration with K. LaMarco and G. Peterson, Tularik, Inc.]

An important focus of our studies on transcriptional regulation has been the structure and function of the VP16-induced complex. It has been known for several years now that the herpesvirus *trans*-activator VP16 does not associate effectively with Oct-1 in the absence of the second host-cell factor HCF. HCF binds to VP16 to form a heteromeric complex and thus primes VP16 for association with Oct-1 in the presence of an appropriate DNA-binding site. Thus, HCF serves an unusual function in the assembly of a multiprotein-DNA complex. Until now, HCF has been relatively poorly characterized, largely because its structure has been unknown.

We have now purified HCF, obtained amino acid sequence, and with this information isolated HCF-encoding cDNAs. The purified protein is a mixture of seven distinct polypeptides. When separated on an SDS-polyacrylamide gel, they form three doublets of approximately 110, 125, and 150 kD and a singlet of about 300 kD. These proteins are apparently all related to one another because the doublet proteins display very similar peptide fingerprints. Unexpectedly, however, the cDNAs we have isolated encode a very large protein of 2035 amino acids, which we believe corresponds to the 300-kD HCF protein. Thus, the smaller proteins are probably derivatives of the 300-kD protein. They are not an artifact of HCF purification, however, because, using HCF antibodies, we

detect the same pattern of HCF polypeptides in extracts from cells that have been rapidly lysed with detergent. We are now focusing on the mechanism by which these smaller polypeptides arise.

Consistent with its unusual function, the deduced sequence of HCF is not related to any other proteins with which we have compared sequences. Within the middle of the predicted HCF sequence, however, there is a set of eight 26-amino-acid-long repeats. These HCF repeats portend the existence of a new type of functional motif. Now that the last piece of the puzzle in the VP16-induced complex is cloned, we can elucidate how it is able to induce VP16 association with Oct-1.

Differential Association of Mammalian Oct-1 Proteins with VP16: An Assay for the Response of Human Oct-1 to VP16 in Murine Cells

M. Cleary

To our surprise, even though the Oct-1 homeodomain is identical in humans, chickens, and *Xenopus*, there is considerable sequence heterogeneity among mammals. For example, as shown in Figure 2A, the murine Oct-1 homeodomain differs from its human counterpart at four positions on the homeodomain

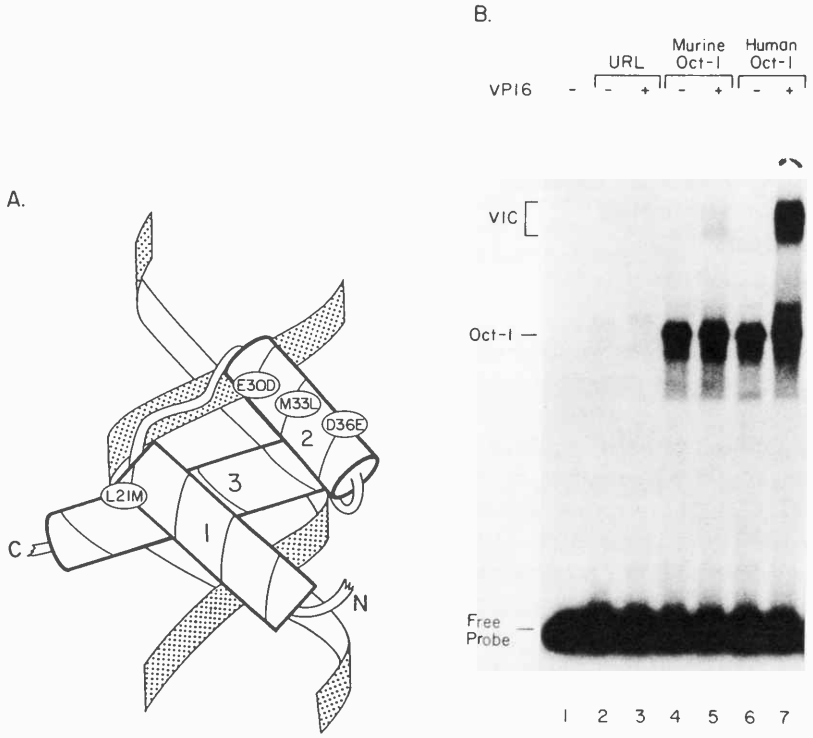

FIGURE 2 Murine Oct-1 associates with VP16 less effectively than human Oct-1. (*A*) The positions and identities of Oct-1 amino acids that differ between the human and murine Oct-1 homeodomains are indicated on an illustration of the *engrailed* homeodomain bound to the major groove of DNA (*stippled*). In the nomenclature system used here, the identity of the human Oct-1 residue is given first in single-letter code, followed by its position in the homeodomain and the identity of the differing murine Oct-1 residue. (*B*) Electrophoretic mobility retardation analysis comparing VP16 association with murine Oct-1 and human Oct-1. Either no in vitro translation extract (lane *1*) or equivalent amounts of unprogrammed (URL; lanes *2,3*), or murine Oct-1 (lanes *4,5*), or human Oct-1 (lanes *6,7*) programmed in vitro translation extracts were incubated with a TAATGARAT probe in the absence (–; lanes *1,2,4,6*) and presence (+; lanes *3,5,7*) of *E. coli*-expressed VP16. All binding reactions contained partially purified human HCF. (VIC) VP16-induced complex. (Reprinted, with permission, from Cleary et al. 1993 [Copyright held by CSHL Press].)

surface responsible for interaction with VP16. Indeed, as shown in Figure 2B, murine Oct-1 does not form a VP16-induced complex (VIC; see lane 5) as effectively as human Oct-1 (lane 7).

We used this difference to establish an in vivo assay for the involvement of human Oct-1 in activation of transcription by VP16. In human cells, everpresent endogenous Oct-1 obscures the activity of transiently expressed Oct-1 from a transfected expression vector. In murine NIH-3T3 cells, however, cotransfection of expression vectors for human Oct-1 and VP16 results in a much larger transcriptional response than with either vector alone. Using this assay, we showed directly that human Oct-1, but not human Oct-2, can respond to VP16 in vivo. The Oct-1 DNA-binding POU domain is sufficient and the Oct-1 homeodomain is critical for this response, because an Oct-1 POU domain containing the Oct-2 homeodomain fails to respond to VP16.

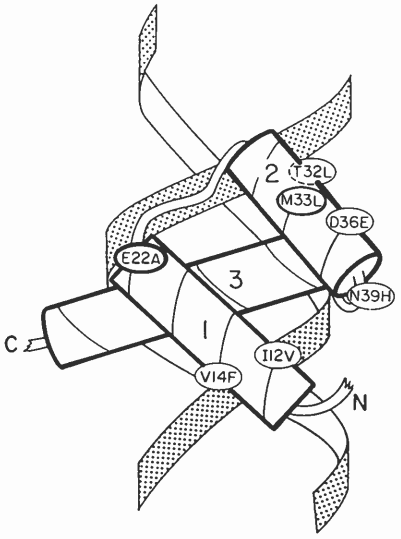

FIGURE 3 The positions and identities of Oct-1 amino acids that differ from the Oct-2 homeodomain are indicated on an illustration of the *engrailed* homeodomain bound to the major groove of DNA (*stippled*). The three homeodomain α-helices (*1–3*) are drawn as bold cylinders. The seven amino acid differences between Oct-1 and Oct-2 homeodomains are circled. The identities and positions of Oct-1 residues are indicated followed by the identities of the amino acids in the Oct-2 homeodomain at the corresponding positions. (N) Amino terminus; (C) carboxyl terminus. (Reprinted, with permission, from Lai et al. 1992 [Copyright held by CSHL Press].)

A Single-amino-acid Exchange Transfers VP16-induced Positive Control from the Oct-1 to the Oct-2 Homeodomain

J.-S. Lai

We have known that the seven differences between the Oct-1 and Oct-2 homeodomains are responsible for the different abilities of Oct-1 and Oct-2 to associate with VP16 and as described above to respond to VP16 in vivo. Figure 3 shows the positions and identities of these seven differences. Now we asked which of these seven differences are key in determining the discrimination between Oct-1 and Oct-2 by VP16. Only two of the differences are important, one in each of the first two α-helices of the tri-α-helical homeodomain. The major determinant is a single glutamic acid residue at position 22 at the top of the first α-helix (circled in bold in Fig. 3). A less significant position is at position 33 (circled with an intermediate thickness). Replacement of the alanine residue found at this position in the Oct-2 homeodomain with the Oct-1-derived glutamic acid residue is sufficient to transfer to Oct-2 the ability to associate with VP16 and respond to VP16 in vivo. Thus, the specificity of positive control of transcription by homeodomains can be conferred by a single-amino-acid difference. This result means that even as little as a single-amino-acid difference on the surface of other homeodomains found in nature can have a significant impact on their ability to regulate transcription.

A T-cell Factor That Recognizes HIV-1 Enhancers Containing Tandem Sequence Duplications

W. Phares

In HIV-1 LTR sequences amplified directly from peripheral blood cells of infected individuals, we have found a hot spot for tandem sequence duplications (TSDs) immediately upstream of the duplicated κB enhancer sequences. These TSDs vary in size from 15 to 29 bp, but all encompass a common 12-bp region. Such duplications in other enhancers, e.g., SV40, affect enhancer function. To analyze their possible significance, we have studied the ability of nuclear proteins to associate with the TSDs in an

electrophoretic mobility retardation assay. We have found an activity in T cells that is absent in the non-T-cell lines we have analyzed. This activity binds to the duplicated forms of the HIV-1 LTR but not the nonduplicated form, and chemical modification interference analysis shows that both copies of the tandemly duplicated sequence are important for association with this T-cell factor. This factor may therefore be responsible for the activity of the TSDs in vivo and will therefore be the focus of our future studies.

Study of Synergism in Transcriptional Activation

M. Tanaka

Studies of the mammalian POU-domain transcription factor Oct-2 have revealed that cooperative interactions among modular elements organized at multiple hierarchical levels are a basis for efficient transcriptional activation. A basic element, an 18-amino-acid subsegment of the Oct-2 glutamine-rich activation domain, was identified by a novel multimerization approach. This 18-amino-acid segment can form a progressively more potent activation domain when tandemly reiterated in increasing numbers. Such an activation domain can cooperatively function with its duplicate or a heterologous proline-rich activation domain on the same polypeptide. When bound to the same promoter, multiples of these activators can cooperate to activate transcription efficiently.

To analyze further the importance of cooperative effects among these modular elements and to investigate a biochemical basis for cooperativity in transcriptional activation process, I plan to use the yeast *Saccharomyces cerevisiae* as a model system and a potent acidic activation domain of HSV VP16 as an activation domain. The multimerization approach was applied to the VP16 activation domain, and several subsegments (between 8 and 13 amino acids in length) were found to activate transcription in yeast when reiterated and fused to the GAL4-DNA-binding domain. I will systematically change a variety of parameters in this model system, including affinity and the number of activator binding sites in a promoter, the number of activation domain subsegments in an activator, and concentration of an activator. Their effects will be examined both in vivo and in vitro for occupancy of activator binding sites as well as efficiency of transcription. Biochemical approaches will further focus on identification and characterization of protein-protein interactions that involve activation domains to develop a mechanistic basis for their synergistic actions.

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MOLECULAR GENETICS OF EUKARYOTIC CELLS

Investigators in this section study diverse biological phenomena, from signal transduction to the pathogenesis of the human immunodeficiency virus. Some of the highlights of the year include the following.

- M. Gilman and colleagues (Nuclear Signal Transduction) have developed an entirely cell-free system which reflects signaling from growth factors to transcriptional activation. Such a system will, in principle, enable the biochemical dissection of an entire signal transduction pathway. They have also made progress characterizing the interaction between the transcription factor SRF and proteins with the homeodomain motif.
- R. Franza and co-workers (Quantitative Regulatory Biology) have continued their studies of cellular regulatory proteins, focusing on proteins that interact with κB sites, and, in collaboration with N. Tonks, protein phosphatases.
- Members of N. Tonks' laboratory (Structure, Function, and Regulation of Protein Tyrosine Phosphatases) have identified novel members of the protein phosphatase family and have characterized many of the biochemical interactions involving these phosphatases. Most significantly, they have shown that a transmembrane receptor-like phosphatase may serve as its own ligand when present on an adjacent cell and thereby mediate cell-cell aggregation.
- D. Bar-Sagi's laboratory (Transmembrane Signaling) continues to study signal transduction pathways in mammalian cells that involve the RAS proteins. They have characterized the proteins upstream of RAS that link its regulation to growth factors. Included among these are novel guanine nucleotide exchange factors and "adaptor" molecules with SH_3/SH_2 domains that link RAS exchange factors with transmembrane protein tyrosine kinases.
- M. Wigler and colleagues (Mammalian Cell Genetics) have continued studies of the RAS signaling pathway. Most significantly, they have identified a protein kinase cascade, conserved from yeasts to humans, that is regulated by RAS. They have shown that RAF (in vertebrates) and byr2 (in fission yeast) are two protein kinases that are likely to be immediate downstream targets of RAS regulation. In addition, the lab has developed a powerful new tool for genomic analysis, called RDA. RDA allows the discovery of new pathogens, the cloning of loci that undergo rearrangement in cancer and sporadic genetic disease, and the isolation of polymorphic probes linked to inherited disease loci. Finally, in collaboration with chemists at Columbia University, the lab has developed a new approach to drug discovery based on combinatorial chemical synthesis and combinatorial decoding.
- S. Patterson's laboratory (Posttranslational Modifications and Apoptosis) has continued to refine methods for the chemical analysis of minute amounts of peptides and has begun an analysis of the changes in protein composition of cells undergoing programmed cell death (apoptosis).
- D. Spector and co-workers (Cell Biology of the Nucleus) have observed changes in protein localization within the nucleus as a consequence of transcriptional activity. In particular, they have observed the shuttling of splicing and other nuclear factors to new sites of RNA transcription.
- The laboratory of D. Helfman (Molecular Cell Biology) continues to study the function and expression of cytoskeletal elements. They have identified new tropomyosins and actins essential for cytokinesis which may work in connection with myosin motors to power cytoplasmic reorganization. They have also identified RNA-binding proteins that are likely to be involved in tissue-specific splicing patterns.

- J. Skowronski and his co-workers (Transgenic Mice) have been using transgenic mice to study the function of proteins encoded by HIV-1, the virus implicated in AIDS. In particular, they have shown that nef down-regulates CDC4 antigen expression on the surface of T cells and alters the development and activation of CDC4$^+$ T cells.
- J. Garrels and colleagues (Quest Protein Database Center) continue the development of software for the analysis of 2D gels and, in collaboration with S. Patterson, R. Franza, and others, the building of databases. A new double amino acid labeling protocol facilitates the identification of 2D protein spots.

TRANSGENIC MICE

J. Skowronski R. Mariani
S. Salghetti
L. Usher

Human immunodeficiency virus (HIV) infection of CD4$^+$ T cells and antigen-presenting cells (APCs) is associated with depletion of CD4$^+$ T cells and deregulation of the immune system that are the hallmarks of the immunodeficient state in AIDS. However, the mechanism(s) by which immunodeficiency viruses subvert the normal function of infected cells and the identity of viral genes that mediate these effects in infected CD4$^+$ T cells are not well understood. Besides the *gag*, *pol*, and *env* genes, found in all retroviruses, immunodeficiency viruses encode several additional "accessory" proteins. The function of some of these accessory genes is not known as they are not required for the viral life cycle in established cell lines. Remarkably, some of these "nonessential" genes are required for efficient viral replication in vivo and for disease induction and therefore have an essential function in the viral life cycle in vivo.

We continue to seek a better understanding of the function and mechanism of action of the nonessential genes of immunodeficiency viruses. Our studies have focused on the *nef* gene of human immunodeficiency virus type 1 (HIV-1). *nef* is dispensable for the viral life cycle in vitro, but although its function is not known, it is strictly required for disease development in primate models of AIDS. Our laboratory has been using approaches in vivo, in transgenic mice, and in vitro to address the consequences of Nef expression on normal cellular functions and its interaction with cellular regulatory mechanisms. In the last year, these studies provided novel and important insights into possible functions of *nef*, which will remain the focus of our studies in the future.

HIV-1 Nef Alters Development and Activation of CD4$^+$ T Cells when Expressed in Transgenic Mice

J. Skowronski, L. Usher, R. Mariani [in collaboration with D. Parks, Stanford University, Stanford]

To assess the consequence of Nef expression in primary cells in vivo, we constructed transgenic mice that express HIV-1 *nef* in T cells. Last year, we reported results of the initial characterization of these CD3 Nef1 transgenic lines. We have shown that CD3 Nef1 animals show abnormally low frequencies of peripheral CD4$^+$ T cells, which is reminiscent of CD4$^+$ T-cell depletion invariably associated with human AIDS. Our recent experiments addressed the mechanism of CD4$^+$ T-cell loss in these transgenic mouse models and the consequences of Nef expression on T-cell receptor function in transgenic T cells.

Analysis of transgene expression patterns in developmental T-cell populations revealed that Nef was expressed at relatively high levels in the immature thymic T cells but not in the mature T cells found on the periphery of transgenic animals. This dichotomy

suggested that Nef may be blocking the normal development of T cells in the thymus and, as a result, T cells that express Nef are depleted from the periphery of transgenic mice. To test this possibility, we characterized developmental populations of thymic T cells in CD3 Nef1 animals. Flow cytometry analysis with monoclonal antibodies to a panel of differentiation antigens revealed that Nef preferentially blocks development of the $CD4^+$ T cells in transgenic animals. These studies also revealed that CD4 antigen expression on both immature and mature transgenic thymic T cells was two to three times lower than that on control thymocytes.

These observations provided an explanation for the defect in T-cell development in CD3 Nef1 mice. CD4 interactions with class II antigens are directly involved in T-cell differentiation. This has been indicated before by findings that development of $CD4^+$ T cells is defective in mice where MHC class II ligands were blocked with monoclonal antibodies or inactivated by a germ-line mutation. Thus, one possible explanation for the defective $CD4^+$ T-cell development in CD3 Nef1 animals is that decreased CD4 expression on immature thymocytes disrupts T-cell receptor MHC class II interactions and hence positive selection of $CD4^+$ T cells. Interestingly, our data are consistent with a rather tight regulation of T-cell development by CD4. It is remarkable that in contrast to previous genetic and antibody-blocking experiments, which virtually eliminated CD4-MHC class II interactions, in CD3 Nef1 mice, the relatively small decrease in CD4 levels on thymic T cells was sufficient to disrupt differentiation of $CD4^+$ T cells.

HIV-1 NEF PROMOTES T-CELL MITOGENESIS

To assess the functional capability of developing T cells, thymocytes isolated from transgenic animals and littermate controls were stimulated with antibodies to CD3 in the presence of phorbol ester. Strikingly, thymocytes from transgenic animals showed elevated responses to stimulation when compared to those of littermate controls (Fig. 1). This effect was remarkable because transgenic thymuses contain a lower fraction of mature T cells than would normally be expected to proliferate in response to this stimulation. In contrast, mitogenic responses to stimulation with phorbol ester and calcium ionophore, which bypasses the T-cell receptor to induce calcium-dependent responses, were not significantly different. This suggested that the observed hyper-

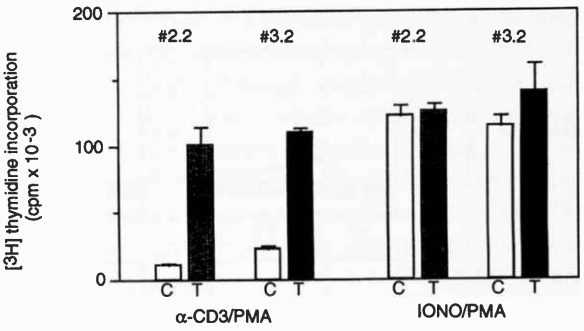

FIGURE 1 Proliferative responses of CD3 Nef1 2.2 thymocytes. Thymic T cells isolated from 2–3-month-old CD3 Nef1 2.2 or CD3 Nef1 3.2 (T) and littermate controls (C) were cultured in the presence of anti-CD3 monoclonal antibody and PMA α-CD3/PMA; 2 μg/ml and 5 ng/ml, respectively) or ionomycin and PMA (IONO/PMA; 0.3 μg/ml and 5 ng/ml, respectively). Proliferation was determined by [^{3}H]thymidine uptake 48 hr after stimulation, and results from triplicate wells are shown. Shown are representatives of four independent experiments.

reactivity of transgenic T cells reflects elevated T-cell receptor signaling.

THYMIC T CELLS ARE HYPER-REACTIVE TO T-CELL RECEPTOR STIMULATION

Accumulation of intracellular calcium is one of the early events following T-cell receptor stimulation. Therefore, to assess T-cell receptor function, we characterized calcium responses in anti-CD3-stimulated thymic T cells (in collaboration with D. Parks, Stanford University). As illustrated in Figure 2, calcium accumulation was elevated in transgenic single positive $CD4^+$ as compared to the control thymocytes. In addition, calcium responses of the immature $CD4^+CD8^+$ T cells were also enhanced. Both the magnitude of the response, which reflects elevated mobilization of free calcium in transgenic cells, and the kinetics of the response were increased. Also, the frequency of responding T cells was almost twofold higher in both the double- and single-positive $CD4^+$ transgenic populations (Fig. 2, two rightmost panels).

DOES NEF ENHANCE T-CELL ACTIVATION OR SURVIVAL ?

What could be the significance of *nef* for viral in vivo replication? Our data indicate that *nef* alters T-cell activation. We suggest that through this effect, *nef* can enhance viral replication in infected T cells. The first possible mechanism is suggested by previous observations indicating involvement of CD4 in the neg-

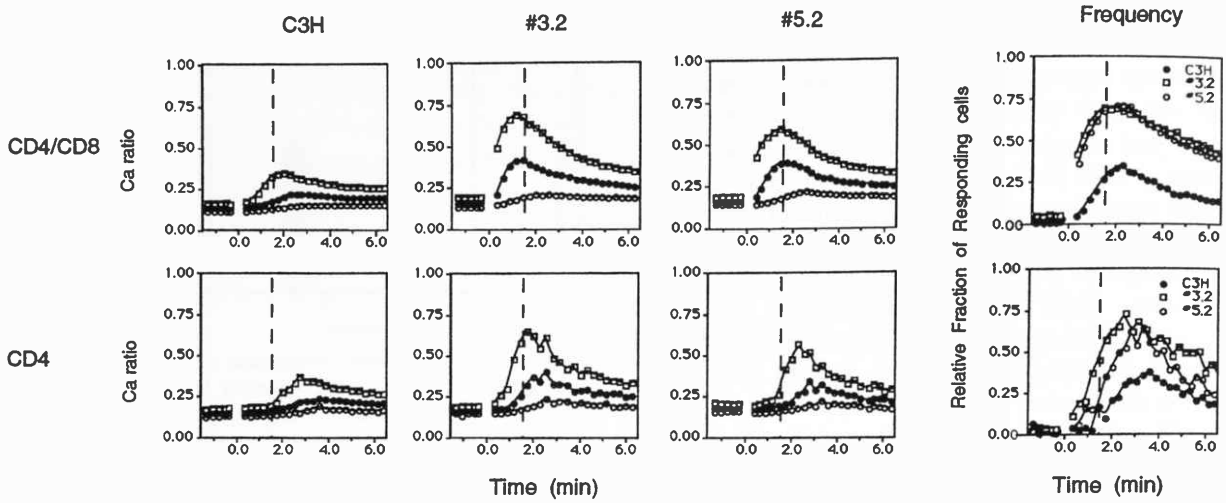

FIGURE 2 Elevated calcium accumulation in CD3 Nef1 thymic T cells. Kinetics of Ca^{++} accumulation in control (C3H), CD3 Nef1 3.2 (3.2) and 5.2 (5.2) thymocytes following stimulation with anti-CD3 monoclonal antibody (145 2C11) was determined by flow cytometry analysis of Indo-1 loaded cells. The distribution of Ca^{++} concentrations in stimulated double-positive (CD4/CD8) and single-positive (CD4) populations is presented as a function of time. Cells were stimulated with 50 μg/ml 145 2C11 monoclonal antibodies at time 0. Each distribution is represented by three curves: The lower curve reflects Ca^{++} ratio at the 20th percentile, the upper curve at 80th percentile, and the middle curve reflects the median Ca^{++} (50th percentile). Calcium concentration is represented by the violet/green ratio of Indo-1 fluorescence (measured with filters at 395–415 nm and 515–560 nm, respectively). All values are normalized to the fluorescence ratio of ionomycin-treated cells (saturating high Ca^{++} concentration + 1.0).

ative regulation of T-cell receptor signaling and activation of mature T cells. Thus, it is conceivable that *nef* may counteract inhibitory effects of CD4 and/or modulate additional signals to promote T-cell activation and viral replication in infected T cells. The second possibility stems from the observation that the cytopathic effect of HIV-1 in cultured T cells involves interaction between CD4 and envelope glycoproteins and may involve a ternary complex with $p56^{lck}$. Although it is not known at present whether *nef* can interact with these complexes, such interactions could result in improved survival of infected $CD4^+$ T cells and augmented viral replication. Either of these effects could be critical for viral survival in the natural setting of immunodeficiency virus infection. Current experiments address these possibilities.

Interaction with CD4 Reflects Conserved Function of Nef

R. Mariani, J. Skowronski

We have observed that NL43 Nef expression results in a low-level CD4 antigen display on the T-cell surface, and down-regulation of CD4 antigen expression on the surface of human T cells has been observed before by other investigators. However, these observations were regarded as controversial because they were observed only with some but not other HIV-1 *nef* alleles. It is well established that *nef* is not required for viral replication in primary T cells nor established T cell lines under the commonly used in vitro conditions, and *nef* open reading frames from HIV-1 and SIV isolates that have been propagated in vitro frequently encode prematurely terminated and hence nonfunctional Nef proteins. Therefore, the apparently contrasting results from in vitro experiments may have reflected genetic variation among commonly studied *nef* alleles; this variation may result from the absence of selection for *nef* function upon in vitro propagation of cloned viruses.

In an attempt to circumvent limitations resulting from the lack of genetic selection for *nef* under the in vitro conditions, we isolated a large number of "primary" HIV-1 *nef* alleles directly from two patients infected with HIV-1 and analyzed the effect of their transient expression on CD4 antigen expression on the surface of human $CD4^+$ CEM T cells. Altogether, we have characterized 15 new HIV-1 *nef* alleles from patient SK1 (NA clones) and 3 from patient SK4 (NB

clones). A compilation of results from these experiments is shown in Figure 3. Expression of Nef from all clones derived from SK1 effected a decrease in CD4 antigen on the surface of CEM T cells, albeit to different extents. Interestingly, *nef* alleles from several of these clones, including NA3, 7, 10, 13, 14, 18, 11, and 15, were more potent than NL43 *nef*. The NA1, 5, 6, 8, and NB1 alleles were as potent as the NL43 Nef. In contrast, the NA4, NA17, NB5, and NB9 alleles were exceptional in that they had little effect on surface CD4 expression; we are currently mapping the loss-of-function mutations in these alleles.

Three lines of evidence from our experiments indicate that CD4 down-regulation reflects a conserved, selected in vivo function of *nef*. First, approximately 80% of all *nef* alleles, including all those that directed detectable expression of Nef protein, down-regulated surface CD4 antigen upon transient expression in CEM T cells. The observation that the remaining 20% of SK1 *nef* alleles showed a weak effect (or no effect) on CD4 is not contradictory. A similar frequency of functionally defective alleles (30%) was observed before with the HIV-1 *tat* gene, which is essential for the viral life cycle, in populations isolated directly from peripheral blood lymphocytes of AIDS patients. Second, although sequence variation observed among primary isolates of Nef proteins and the NL43 Nef amounted altogether to more than 40 amino acid substitutions, most reflected conservative changes with little or no detectable effect on CD4 down-regulation. Third, CD4 down-regulation was disrupted by mutations in amino acid motifs that are conserved among human and simian Nef proteins. Remarkably, an intact amino-terminal myristoylation site, which is invariantly found in both HIV-1 and HIV-2/SIV Nef proteins, was required for full Nef activity. These results provided a stage for current experiments to address the biochemical mechanism(s) of CD4 down-regulation by Nef.

PUBLICATIONS

Rothstein, J.L., D. Johnson, J. DeLoia, J. Skowronski, D. Solter, and B. Knowles. 1992. Gene expression during preimplantation mouse development. *Genes Dev.* **6:** 1190–1201.

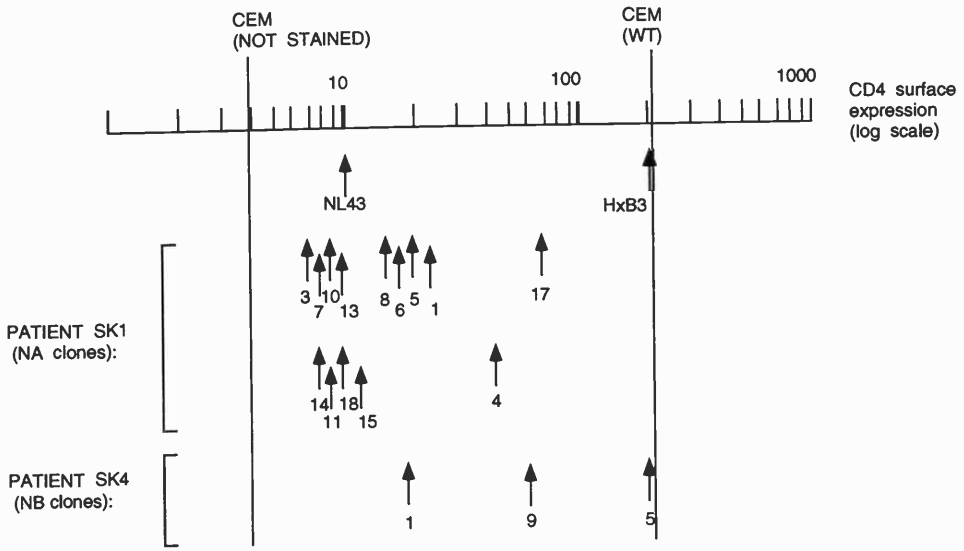

FIGURE 3 Primary HIV-1 and SIV Nef isolates down-regulate CD4 in human CD4$^+$ T cells. Compilation of results from flow cytometry analyses of *nef* alleles from patients SK1 and SK4. Each arrow represents an individual NA or NB *nef* clone, which is identified by a number below. Each arrow reflects the median level of CD4 expression on the surface of CEM cells transfected with the respective expression vector. Vertical lines represent the wild-type level CD4 expression on CEM T cells (CEM wt). CD4 expression on CEM cells electroporated with the CD3 NL43 (which is associated with abnormally low CD4 expression in CD3 Nef1 transgenic mice) and CD3 HxB3 (which encodes a nonfunctional Nef protein) are shown for comparison.

In Press, Submitted, and In Preparation

Mariani, R. and J. Skowronski. 1993. CD4 downregulation by *nef* alleles isolated from HIV-1 infected individuals. *Proc. Natl. Acad. Sci.* (in press).

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MAMMALIAN CELL GENETICS

M. Wigler R. Ballester, E. Chang, K. Sen, K. O'Neill, G. Asouline, H.-P. Xu, J. Camonis, A. Polverino, M. Barr, N. Lisitsyn, N. Lisitsyn, L. Van Aelst, R. Swanson, L. Rodgers, J. Brodsky, G. Bolger, S. Marcus, C. Nicolette, M. Riggs, J. Douglas, T. Michaeli, V. Jung

This was an extraordinary year. For the 12-month period ending as of this writing, three lines of work culminated in extremely important achievements in diverse areas of molecular biology. First, our studies of *RAS* oncogene function have led to the discovery that *RAS* activates a protein kinase cascade, conserved in evolution from yeast to metazoans. This activation is probably mediated through a direct interaction with a MAP protein kinase kinase kinase (MAPKKK). In mammalian cells, one such MAPKKK upon which *RAS* acts is likely to be the RAF oncoprotein. In *Schizosaccharomyces pombe*, a fission yeast, it is the byr2 kinase. The methodologies we have employed in these studies, cross species complementation and the S. Fields and O.-K. Song genetic system to detect protein/protein interactions, are likely to yield yet additional yeast and mammalian elements that operate in this pathway. Second, we have developed a major new genetic tool that detects the differences between two like DNA populations. This tool (RDA) enables us to discover probes for pathogenic genomes from infected tissues, to clone probes corresponding to the genetic lesions from neoplastic cells and from organisms suffering spontaneous genetic disease, and to clone probes linked to inherited disease loci. Third, in collaboration with Clark Still and his colleagues in the Department of Chemistry at Columbia University, we have developed the most powerful tool yet for conducting combinatorial chemistry. This tool allows the synthesis of extremely large numbers of chemical compounds, the selection of the few with desirable characteristics, and the determination of the structure of those few. In short, the combinatorial methods we have developed enable organic chemists to operate like geneticists. With such methods, the discovery of small molecular agonists and antagonists to desired targets may become as easy as raising monoclonal antibodies.

Regulation of RAS

R. Ballester, L. Van Aelst, J. Camonis, V. Jung

RAS genes encode GTP/GDP-binding proteins, found in yeasts and metazoans. They were first discovered as the oncogenes of acutely transforming retroviruses and then rediscovered as mutated alleles of the first human oncogenes. Wild-type RAS proteins participate in physiologically normal signal transduction pathways that regulate growth and differentiation in a wide variety of cell types. Signaling from RAS occurs in its GTP-bound state, and normal RAS protein slowly hydrolyzes GTP. The wild-type proteins are regulated by at least two types of molecules: factors that accelerate guanine nucleotide exchange on RAS and factors that accelerate guanine nucleotide triphosphate hydrolysis by RAS. CDC25 of the yeast *Saccharomyces cerevisiae* was the first protein of the first class to be discovered (Camonis et al., *EMBO J. 5:* 375 [1986]; Broek et al., *Cell 48:* 789 [1987]). Mammalian GAP was the first protein of the second class to be discovered (Trahey and McCormick, *Science 238:* 542 [1987]). Both CDC25 and GAP are

representatives of large families of proteins, found in a wide variety of eukaryotic organisms. Mutant, oncogenic RAS proteins are frequently abnormal in their regulation, and in particular in their response to GAP. The object of this section has been to understand the nature of the components and pathways that regulate RAS.

Work in *S. cerevisiae* has indicated the existence of at least three CDC25-like molecules: CDC25 itself, BUD5 (Chant et al., *Cell 65:* 1213 [1991]; Powers et al., *Cell 65:* 1225 [1991]), and SDC25 (Boy-Marcotte et al., *Gene 77:* 21 [1989]; Damak et al., *Mol. Cell. Biol. 11:* 202 [1991]). Previous genetic analysis suggested a role only for CDC25 in the physiological regulation of RAS. The biochemical role of CDC25 was clarified by the discovery of dominant interfering forms of *RAS2*, RAS^{Gly22}, and RAS^{Ala15}, which block CDC25 function (Powers et al., *Mol. Cell. Biol. 9:* 390 [1989]). BUD5 appears likely to regulate BUD1, which is a RAS-like protein (also known as RSR1) involved in polarity selection during bud formation (Bender and Pringle, *Proc. Natl. Acad. Sci. 86:* 9976 [1989]; Chant and Herskowitz, *Cell 65:* 1203 [1991]). The true physiological role of SCD25 is unknown. In high copy, a truncated *SCD25* gene can complement the loss of *CDC25*, but the disruption of *SCD25* has no apparent phenotype.

Molecular analysis of CDC25 has revealed clues about its regulation. The smallest domain of CDC25 needed for viability is a carboxy-terminal portion (from amino acid 1102 to 1541). The entire molecule is 1589 amino acids long. Regulatory regions are found in the amino-terminal domains. A point mutation (S 364 F) activates CDC25, and overexpression of an amino-terminal domain (from amino acid 1 to 239) blocks wild-type CDC25 function. Cells expressing only the minimal catalytic region of CDC25 still exhibit glucose-dependent cAMP regulation (Van Aelst et al., *Eur. J. Biochem. 193:* 675 [1990]; R. Ballester and L. Van Aelst, unpubl.). Therefore, either this minimal region contains the regulatory domains for glucose responsiveness or the pathway that regulates cAMP in response to glucose does not pass through CDC25. One interesting possibility is that glucose regulates adenylyl cyclase through GPA2, a protein that is in the family of the G_α subunits of heterotrimeric G proteins (Nakafuku et al., *Proc. Natl. Acad. Sci. 85:* 1374 [1988]). GPA2 has been shown to affect cAMP levels and is a homolog of gpa2, an *S. pombe* protein that is known to regulate adenylyl cyclase (Isshiki et al., *Genes Dev. 6:* 2455 [1992]). If this hypothesis is correct, then RAS proteins and GPA2 may codominantly regulate adenylyl cyclase. The codominance of RAS proteins and G proteins is a theme to which we will return.

In *S. pombe*, there is a single known RAS homolog, ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]). It is regulated by a CDC25-like protein called ste6 (Hughes et al., *Nature 344:* 355 [1990]). Whereas ras1 is required for cell shape, sporulation, and conjugation, ste6 is required only for conjugation. Therefore, other nucleotide exchange regulators of RAS may be found in *S. pombe*. We have obtained direct evidence for this. Upon testing libraries of mutated *ras1* genes, we found several dominant negative *ras1* mutants. One of these ($ras1^{Tyr62}$) induced a *ras1* null phenotype when overexpressed in wild-type cells but had minimal effects when overexpressed in cells containing the activated $ras1^{Val17}$ gene. We therefore presume that $ras1^{Tyr62}$ blocks the activation of ras1 by acting upon a regulatory molecule other than ste6. One candidate for this is ral2. Disruption of *ral2* produces a phenotype that is very similar to the *ras1* null phenotype (Fukui et al., *Mol. Cell. Biol. 9:* 5617 [1989]). However, the primary sequence of ral2 does not resemble that of CDC25-like molecules. Preliminary data, obtained in collaboration with Dan Broek, indicate that RAS molecules with the Tyr-62 mutation bind irreversibly to CDC25-like molecules.

Genes with regions homologous to the catalytic domain of CDC25 have been found in flies (Sos) and mammals (rasGRF, mSos1, mSos2) (Simon et al., *Cell 67:* 701 [1991]; Botwell et al., *Proc. Natl. Acad. Sci. 89:* 6511 [1992]; Shou et al., *Nature 358:* 351 [1992]). Although rasGRF has been shown to catalyze guanine nucleotide exchange on RAS (Jacquet et al., *J. Biol. Chem. 267:* 24181 [1992]; Shou et al., *Nature 358:* 351 [1992]), this had not been shown for the Sos proteins. In collaboration with Pierre Chardin and Dafna Bar-Sagi, we demonstrated that a fragment (amino acids 707–1059) of hSos1, a human homolog of *Drosophila* Sos and of mouse Sos1, complements the loss of CDC25 in *S. cerevisiae* and stimulates nucleotide exchange on RAS in vitro. Extracts from mammalian cells where hSos1 is overexpressed exhibit higher nucleotide exchange activity on RAS. These data demonstrate that hSos1 is a bona fide nucleotide exchange catalyst for RAS. Larger fragments of hSos1 failed to complement a loss-of-function mutation of CDC25, suggesting the exis-

tence of negative regulatory domains. Further studies will center upon the regulation of hSos1 function.

Recently a gene, *SEM5*, has been identified in *Caenorhabditis elegans*, a simple worm, that encodes a protein that appears to act upstream of RAS (Clark et al., *Nature 356:* 340 [1992]). Homologs of this gene have been found in flies and mammals (Lowenstein et al., *Cell 70:* 431 [1992]). In mammals, the gene is called *GRB2*. This gene encodes a protein composed entirely of SH3 and SH2 domains, in the order SH3/SH2/SH3. SH2 domains, which are found on a wide variety of molecules involved in signal transduction, bind certain peptide motifs containing phosphotyrosine (Pawson and Gish, *Cell 71:* 359 [1992]). SH3 domains are also found on a wide variety of signal transduction molecules, and bind certain peptide motifs, but these motifs are less well characterized (Ren et al., *Science 259:* 1157 [1993]). Two of the human homologs of CDC25, hSos1 and hSos2, contain peptides in their carboxy-terminal domains that are candidate SH3-binding motifs. We therefore reasoned that the Sos proteins might bind to GRB2. To test this, we utilized a genetic assay in *S. cerevisiae* for protein/protein interactions, the two hybrid system of Fields and Song (*Nature 340:* 245 [1989]). These studies indicated that GRB2 interacts with the carboxy-terminal domain of hSos1 (amino acids 1131 to 1333), and this was further demonstrated by direct coprecipitation. Our genetic studies indicated that both SH3 domains are required for interaction.

We have continued our investigations of NF1 in an ongoing collaboration with Francis Collins and his colleagues at the University of Michigan. *NF1* is the locus for Von Recklinghausen's neurofibromatosis, an inherited disease affecting the proliferation and differentiation of neuroepithelium. It encodes a member of the GAP family of GTPase-activating proteins capable of causing the down-regulation of RAS proteins (Xu et al., *Cell 62:* 599 [1990]; Wang et al., *Cell Regul. 2:* 453 [1991]). *NF1* mRNA comes in two alternately spliced forms, one with an insertion in the midst of the region thought to encode the catalytic domain (Anderson et al., *Mol. Cell. Biol. 13:* 487 [1993]). Both spliced forms are expressed in all tissues examined, although in varying ratios. We demonstrated that both forms are catalytically active. Mutational studies of the conserved residues of NF1 have indicated that certain are required for full activity, as judged by genetic assays in *S. cerevisiae* (Gutmann et al., *Oncogene 8:* 761 [1993]). Substitution at residue 1426 abolishes activity, and substitutions at residues 1423 and 1391 merely diminish activity.

Questions remain whether GAP-like molecules have RAS-effector function. The evidence is clear that GAP can down-regulate RAS, but evidence is also accumulating that GAP has some effector activity (Yatani et al., *Cell 61:* 769 [1990]; Martin et al., *Science 255:* 192 [1992]; Schweighoffer et al., *Science 256:* 825 [1992]). We have continued to pursue this issue for the *IRA* genes, which encode GAP-like molecules in *S. cerevisiae* (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]). Disruption of both *IRA* and *IRA2* in *S. cerevisiae* leads to the same phenotype seen when *RAS2* is activated by mutation. Disruption of *RAS2* in an *ira1*$^-$ *ira2*$^-$ background restores a wild-type phenotype. Thus, the only function we can detect for the IRA proteins is the down-regulation of RAS protein (R. Ballester, unpubl.).

A Conserved Cascade of Protein Kinases: RAS Effector Function

S. Marcus, L. Van Aelst, A. Polverino, H.-P. Xu, M. Barr, M. White, K. Sen

In the fission yeast *S. pombe*, ras1 participates in pheromone-induced sexual differentiation (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis, *EMBO J. 5:* 2963 [1986]; Nielson et al., *EMBO J. 11:* 1391 [1992]; Neiman et al., *Mol. Biol. Cell 107:* [1993]). *ras1*$^-$ cells are unable to conjugate, and they sporulate inefficiently. *gpa1*, a gene that encodes a protein homologous to the G_α subunits of heteromeric G proteins, is also required for conjugation and sporulation (Obara et al., *Proc. Natl. Acad. Sci. 88:* 5877 [1991]). *gpa1* presumably mediates the signaling from the pheromone receptors. Studies during the past few years have indicated that overexpression of several genes is capable of bypassing the requirement for ras1 for sporulation in *S. pombe*. Among these genes are *byr2* and *byr1*, two genes encoding putative protein kinases and also required for both conjugation and sporulation (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]; Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]). Overexpression of these genes also restored sporulation to *gpa1*$^-$ cells (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). Epistasis experiments, utilizing sporulation of diploid cells as the observable

phenotype, placed byr1 downstream from byr2, and both downstream from ras1 and gpa1, but these experiments could not order ras1 and gpa1 with respect to each other.

We have succeeded in ordering gpa1 and ras1 by examining expression of *mam2*, the gene encoding the receptor for P-factor pheromone (H.-P. Xu et al., submitted). We have found that mRNA levels for *mam2* depend on components of the pheromone signaling pathway itself. We view this relationship as a rudiment of a sexual arousal response. Disruption of *ras1*, *gpa1*, *byr1*, or *byr2* reduces *mam2* expression 10- to 100-fold in wild-type homothallic cells. By overexpressing these genes, we have confirmed that byr1 and byr2 act downstream from gpa1 and ras1 but also that gpa1 acts downstream from ras1, and ras1 downstream from gpa1. In other words, ras1 and gpa1 are codominant. These same studies find no role for spk1 in the regulation of *mam2* (see below).

Work continues to find other elements of *S. pombe* that participate in the sexual differentiation pathway. We have found mutants of diploid *S. pombe* that sporulate in the absence of ras1 and have demonstrated that one of these mutants is recessive. The mutant locus encodes a product that appears to act upstream of byr2. Hence, the mutant locus might encode a protein that normally functions to block sexual differentiation, perhaps by acting on the pathway between gpa1 and byr2 or between ras1 and byr2. In a similar approach, we have constructed *S. pombe* strains containing a *mam2* promoter-reporter gene (*ADE2*) that we are using to look for new mutations in the sexual differentiation pathway.

In collaboration with Aaron Nieman in Ira Herskowitz's laboratory at the University of California, San Francisco, and Brian Stevenson in the laboratory of George Sprague, University of Oregon, we showed that the byr2 and byr1 kinases are each structurally and functionally related to the STE11 and STE7 kinases of *S. cerevisiae*, respectively (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). STE11 and STE7 are themselves required for the pheromone-induced sexual differentiation pathways in the budding yeast, with STE11 acting upstream of STE7 (Teague et al., *Proc. Natl. Acad. Sci. 83:* 7371 [1986]; Rhodes et al., *Genes Dev. 4:* 1862 [1990]; Cairns et al., *Genes Dev. 6:* 1305 [1992]; Gartner et al., *Genes Dev. 6:* 1280 [1992]; Stevenson et al., *Genes Dev. 6:* 1293 [1992]). Our studies of cross species complementation suggest that byr1 and byr2 cooperate in *S. cerevisiae*, and hence probably directly interact. Furthermore, byr2 appears to restore pheromone responsiveness to STE11-deficient cells, indicating that the regulation of byr2 by upstream elements has been conserved.

A pair of protein kinases, FUS3 and KSS1, found in *S. cerevisiae*, also participate in the sexual differentiation pathway, probably downstream from STE11 and STE7 (Gartner et al., *Genes Dev. 6:* 1280 [1992]; Errede et al., *Nature 362:* 261 [1993]). They are homologous to the spk1 protein kinase of *S. pombe*, likewise required for sexual differentiation in that organism (Toda et al., *Genes Dev. 5:* 60 [1991]; Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). In our collaborative studies, we demonstrated that spk1 and the FUS3/KSS1 kinases share function. Most significantly, these kinases are structurally related to the MAP/ERK protein kinases of vertebrate cells (Boulton et al., *Cell 65:* 663 [1991]; Toda et al., *Genes Dev. 5:* 60 [1991]). The latter kinases are activated, in a RAS-dependent fashion, by a variety of extracellular factors (DeVries-Smits et al., *Nature 357:* 602 [1992]; Robbins et al., *Proc. Natl. Acad. Sci. 89:* 1624 [1992]; Thomas et al., *Cell 68:* 1031 [1992]; Wood et al., *Cell 68:* 1041 [1992]). This suggested a possible conserved kinase cascade between yeasts and vertebrates. Consistent with this, we demonstrated that ERK2, a member of the MAP kinase family, could partially replace spk1 function in *S. pombe* (Neiman et al., *Mol. Cell. Biol. 4:* 107 [1993]). Significantly, a MAP kinase kinase, called MEK, was found by others that is homologous to STE7 and byr1 (Crews et al., *Science 258:* 478 [1992]) and, even more recently, a MEK kinase (MEKK) that is homologous to STE11 and byr2 (Lange-Carter et al., *Science 260:* 315 [1993]).

The dependence of agonist-induced MAP kinase activation upon RAS was demonstrated by other investigators who showed that interfering forms of RAS block MAP kinase activation (DeVries-Smits et al., *Nature 257:* 602 [1992]; Robbins et al., *Proc. Natl. Acad. Sci. 89:* 1624 [1992]; Thomas et al., *Cell 68:* 1031 [1992]; Wood et al., *Cell 68:* 1041 [1992]). Moreover, scrape loading cells with mutant, activated RAS leads to MAP kinase activation (Leevers and Marshall, *EMBO J. 11:* 569 [1992]). In collaboration with Ellen Shibuya of Joan Ruderman's laboratory at Harvard Medical School, we showed that this interaction could be observed in cell-free *Xenopus* oocyte extracts (Shibuya et al., *Proc. Natl. Acad. Sci. 89:* 9831 [1992]). Addition of activated RAS protein results in a slow (1–3 hr) activation of MAP kinase.

Mutant RAS that lacks the site for carboxy-terminal processing failed to activate MAP kinase. The slow kinetics of the response to activated RAS probably reflects the slow processing and maturation of RAS.

In vertebrates, the RAF oncoprotein is a candidate downstream effector of RAS. RAF can overcome growth defects in cells in which RAS function is blocked (Smith et al., *Nature 320:* 540 [1986]; Cai et al., *Mol. Cell. Biol. 10:* 5314 [1990]). Interfering forms of RAF block RAS function (Kolch et al., *Nature 349:* 426 [1991]). Moreover, RAF is capable of phosphorylating MEK, a MAP kinase kinase (Dent et al., *Science 257:* 1404 [1992]; Howe et al., *Cell 71:* 335 [1992]; Kyriakis et al., *Nature 358:* 417 [1992]), and RAF becomes hyperphosphorylated in response to RAS activation (Morrison et al., *Proc. Natl. Acad. Sci. 85:* 8855 [1988]; Wood et al., *Cell 68:* 1041 [1992]). To begin our investigation of the relationship between RAS and RAF, we expressed RAF in *S. pombe*. We observed inhibition of sexual conjugation by RAF and partial suppression of the phenotypes of activated RAS. No phenotype was observed when we expressed an "activated" RAF, lacking the amino-terminal, putative regulatory domain. These experiments suggested that interactions with upstream elements regulating RAF may have been conserved.

We decided to test directly whether RAS and RAF could interact. To do this, we employed a genetic system in *S. cerevisiae*, Fields and Song's (*Nature 340:* 245 [1989]) two hybrid system, that detects physical complex formation between proteins fused to the separated activating and binding DNA domains of the transcriptional activator GAL4. These experiments revealed that RAS forms a complex with RAF (L. Van Aelst et al., *Proc. Natl. Acad. Sci.* [in press]). RAS mutated in its effector loop, or in the domain required for GTP binding, would not interact with RAF. Binding was to the amino terminus of RAF, believed to be its regulatory domain. These experiments give further evidence for the hypothesis that RAF is a downstream effector of RAS and suggest that RAF is an immediate downstream effector. At present, we cannot rule out the possibility that the interactions between RAS and RAF are mediated by a third protein.

We further showed that RAF can form a complex with MEK, again using the two hybrid system. This interaction requires only the catalytic domain of RAF. Moreover, RAS can form a complex with MEK, but only if RAF is also overexpressed. This suggests either that RAF forms a protein bridge between RAS and MEK or, less likely in our view, that RAF alters MEK so that it can bind to RAS. It is now easier to formulate possible hypotheses about how RAS regulates RAF function: RAS might directly activate RAF or RAS might cause RAF to colocalize with RAS, bringing RAF in proximity to other regulators or substrates. These comments bear upon MEK regulation and even MAP kinase regulation by RAS.

Our results with RAF encouraged us to examine complex formation between RAS and byr2. byr2 plays a role in *S. pombe* not unlike the role of RAF in mammalian cells. byr2 appears to mediate signals from RAS and appears to activate byr1 (Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]; Neiman et al., *Mol. Biol. Cell 4:* 107 [1993]), which is homologous to MEK. Again, we found that RAS could form a complex with byr2, and again with its amino-terminal, putative regulatory domain. Although byr2 has an overall similarity to RAF in that it is a large protein kinase with a catalytic carboxy-terminal domain, outside this domain it has no evident primary sequence homology with RAF. Moreover, neither RAF nor byr2 bear any resemblance to the GAP family, proteins known to interact with RAS. Thus, we think that either a small and as yet unrecognized primary sequence motif is responsible for RAS interaction or interaction with RAS requires a conformational motif.

Conjugation and Shape in *S. pombe*

E. Chang, J. Camonis

While attempting to define genes that might act on the RAS pathway, we looked for mutations that produced phenotypes similar to those of *ras1*$^-$ cells. Several mutants were obtained that were both round and sterile, and the loci responsible were identified. These were called *scd1* and *scd2* (for *s*hape and *c*onjugation *d*eficient). Sequence analysis indicates that scd1 is globally homologous to *S. cerevisiae* CDC24 (Miyamoto et al., *Gene 54:* 125 [1987]), whereas scd2 is closely related to *S. cerevisiae* BEM1 (Chenevert et al., *Nature 356:* 77 [1992]). Moreover, *CDC24* can partially complement loss of *scd1* function. CDC24 and BEM1 participate in bud formation

and bud site selection in *S. cerevisiae* (Bender and Pringle, *Mol. Cell. Biol. 11:* 1295 [1991]; Chant et al., *Cell 65:* 1213 [1991]). In *S. cerevisiae*, this process involves at least two members of the RAS superfamily: BUD1 (Bender and Pringle, *Proc. Natl. Acad. Sci. 86:* 9976 [1989]; Chant and Herskowitz, *Cell 65:* 1203 [1991]), also known as RSR1, which is closely related to the RAS proteins, and CDC42, which is a member of the RHO subfamily. We tested the interrelationship of these gene products and have found that *S. pombe ras1* (but not *S. cerevisiae RAS2*) can suppress defects in *S. cerevisiae CDC24*, and a member of the mammalian RHO subfamily, CDC42Hs (Shinjo et al., *Proc. Natl. Acad. Sci. 87:* 9853 [1990]), which can suppress the loss of *CDC42* in *S. cerevisiae*, can suppress the morphological defects of *ras1*$^-$ *S. pombe*. We derive two hypotheses from these observations. The first is that common molecular mechanisms can be adapted to different morphogenic programs (budding in *S. cerevisiae*, shape and conjugation in *S. pombe*). The second is that ras1 in *S. pombe* may have a role that is homologous to both *S. cerevisiae* RAS and *S. cerevisiae* BUD1. In other words, whereas *S. cerevisiae* has evolved two diverged forms of RAS, one specialized for regulating morphogenesis (budding) and one specialized for signal transduction (through the cAMP-dependent protein kinases), *S. pombe* has a single form of RAS capable of regulating both morphogenesis (cell shape and conjugation) and signal transduction (the MAP protein kinase cascade).

The Function of CAP and Related Proteins

K. O'Neill

We previously described the adenylyl-cyclase-associated protein (CAP) required for RAS-stimulated adenylyl cyclase activity in *S. cerevisiae* (Field et al., *Cell 61:* 319 [1990]; Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]). The *S. pombe* homolog, cap, is also associated with adenylyl cyclase and appears to be required for its proper regulation (Kawamukai et al., *Mol. Cell. Biol. 3:* 167 [1992]), although the regulator of adenylyl cyclase in *S. pombe* does not appear to be ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis and Nasim, *Mol. Cell. Biol. 10:* 549 [1990]; Kawamukai et al., *Cell Regul. 2:* 155 [1991]). In *S. pombe*, the *gpa2* gene product, a G_α-like protein, appears to be required for adenylyl cyclase regulation (Isshiki et al., *Genes Dev. 6:* 2455 [1992]). CAP proteins are bifunctional proteins, as we have described in previous years (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]; Kawamukai et al., *Mol. Cell. Biol. 3:* 167 [1992]). The amino terminus is associated with adenylyl cyclase and the carboxyl terminus is associated with morphological functions.

Recently, in collaboration with Makoto Kawamukai, formerly from this lab but now returned to Shimane University, in Japan, we have succeeded in cloning a human homolog of CAP by complementation in *S. pombe* of the loss of carboxy-terminal cap function. This gene encodes a protein that is the homolog of a porcine protein, called ASP-56, isolated from platelets based on its actin-binding properties (Gie-selmann and Mann, *FEBS Lett. 298:* 149 [1992]). In collaboration with Jeff Field, we have shown that both the human and *S. cerevisiae* CAP proteins can bind to *S. cerevisiae* actin. We previously noted that loss of carboxy-terminal CAP function in *S. cerevisiae* could be suppressed by overexpression of profilin, an actin- and phospholipid-binding protein (Vojtek et al., *Cell 66:* 497 [1991]). This leads us to postulate that the carboxyl terminus of CAP and profilin have redundant function. CAP homologs have now been found in worms and hydra, and a second human CAP, CAP2, has been found (collaborations with M. Chalfie, Columbia University, New York, and C. Schaller, University of Hamburg, Germany). The carboxy-terminal portion is the only portion that appears to be so conserved that it functions in cross species complementation. However, the amino-terminal primary structure is at least partially conserved. This raises our hopes that the amino-terminal domains bind adenylyl cyclase in other species, and we are hopeful of discovering a homolog of the yeast form of this enzyme in mammals.

Analysis of the Mechanism of Tumor-promoting Phorbol Esters and Protein Kinases C

C. Nicolette

In previous years, we identified a number of human cDNAs that, when expressed in *S. cerevisiae*, could

block the phenotypes induced by activated RAS (Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). One of these, called JC310, has proven to be of particular interest because its overexpression in mammalian cells has a striking phenotype. Analysis indicates that expression of JC310 blocks the action of the tumor-promoting phorbol esters. Phorbol esters activate protein kinases C (PKC), which are thought to mediate the action of the phorbol esters. In particular, phorbol esters induce DNA synthesis in quiescent cells, induce morphological change, induce phosphorylation of PKC substrates, induce phosphorylation of MAP kinase, and induce *fos* expression (Kikkawa and Nishizuka, *The Enzymes*, Academic Press [1986]; Ono et al., *J. Biol. Chem. 263:* 6927 [1988]).

Cells selected for overexpression of JC310 are resistant to phorbol-ester-induced DNA replication, *fos* expression, and morphologic change. However, such cells do undergo the changes in protein phosphorylation induced by the phorbol esters. Cells transiently expressing JC310 and a *fos-CAT* reporter construct do not respond to phorbol esters by induction of CAT (chloramphenicol acetyltransferase). Expression of JC310 appears to have no effect on the cellular response to RAS, growth factors that are mediated through tyrosine kinase receptors, or serum. We have not yet tested factors that act through G proteins. JC310 localizes to the nucleus.

These results require some rethinking about phorbol-ester- and RAS-induced signaling. First, our results throw into question the cooperative, interdependent relationship between RAS and PKCs (Yu et al., *Cell 52:* 63 [1988]; Gauthier-Rouviere et al., *EMBO J. 9:* 171 [1990]). Moreover, JC310 blocks phorbol esters without apparently blocking the activation of PKC, and hence phorbol esters might induce effects through a previously uncharacterized route. Alternately, JC310 blocks nuclear signaling from the PKC. The further implication is that nuclear signaling from phorbol esters proceeds through a "private line" of communication with the nucleus that is not shared by serum, RAS, or growth factors acting via tyrosine kinase receptors. Detailed analysis of the regulation of the *fos* promoter are consistent with this hypothesis (Gilman, *Genes Dev. 2:* 394 [1988]; Cai et al., *Mol. Cell. Biol. 10:* 5314 [1990]). Finally, the failure of cells that overexpress JC310 to respond to the morphological effects of phorbol esters brings into question the belief that these rapid effects are mediated by the purely cytoplasmic effects of activating PKC.

Characterization of Mammalian cAMP Phosphodiesterases

T. Michaeli, G. Bolger

Several years ago, we reported that mammalian genes encoding cAMP phosphodiesterases (PDE) could be cloned by selecting from mammalian cDNA libraries those genetic elements that could block the effects of activated RAS ($RAS2^{Val19}$) in *S. cerevisiae* (Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]). Our first PDEs were homologous to the *Drosophila dunce* gene involved in learning and memory in fruit flies. We now know that there are four such genes in humans and that they encode high affinity, cAMP-specific PDEs that are rolipram-sensitive. Sequence analysis indicates that they share two regions of conserved sequence upstream of the catalytic region (UCR1 and UCR2) and some of the same splice junctions with the *Drosophila dunce* PDE (G. Bodger et al., submitted).

We have also identified a new human PDE, representing a novel family of PDEs, class VII, that we have called HCP. HCP is a very high affinity (K_m ~0.1 μM) cAMP-specific PDE that is insensitive to both milrinone and rolipram. Its expression is especially high in skeletal muscle, but we find it is also expressed in the heart and brain. HCP was cloned by suppression of the phenotype of a $pde1^-$ $pde2^+$ *S. cerevisiae* strain. This latter strain is a more sensitive host strain for screening PDEs than is the $RAS2^{Val19}$ strain (T. Michaeli et al., *J. Biol. Chem.*, in press.)

Difference Cloning

N. Lisitsyn, N. Lisitsyn, H.-P. Xu

In 1981, it was apparent that gene transfer was detecting mainly mutant *RAS* genes in tumor cells and that an unbiased method was required for the detection of genetic lesions in tumors. In 1985, we began in earnest to develop a "difference cloning" methodology that would enable us to detect the differences between two complex genomes. Our early efforts culminated in a method that could achieve 100–1000-fold enrichment of "difference" sequences (i.e., "target" found in the "tester" genome, but not present in the "driver" genome) by reiterated subtractive methods (Wieland et al., *Proc. Natl. Acad. Sci.*

87: 2720 [1990]). We also realized that the second-order kinetics of DNA reannealing could be harnessed to achieve even greater enrichment, but we could not combine the subtractive and kinetic steps. Hence, we failed to achieve the levels of enrichment required for the difference analysis of complex genomes.

In 1991, Nikolai Lisitsyn arrived at Cold Spring Harbor to continue studies that he had begun on this problem in Moscow. He brought with him an elegant technique, based on the polymerase chain reaction (PCR), to amplify small quantities of homoduplex DNA in the presence of large quantities of unannealed DNA or heteroduplex DNA. While here, we engineered this technique into a powerful difference analysis method that utilized subtractive and kinetic enrichment together in each repetitive step. In the analysis of complex genomes, we found it was necessary first to sample the genomes, reducing their complexity in a reproducible way. We achieved this by "whole genome" PCR in which the DNA is cleaved, ligated to template oligonucleotide linkers, and then amplified by PCR. Only small fragments amplify well and hence the resulting "amplicon" has a smaller overall complexity than the starting material. Through multiple samplings, much of one genome can thus be scanned for differences with another genome. The details of the method, which we call representational difference analysis (or RDA), were recently published (Lisitsyn et al., *Science 259:* 946 [1993]). We demonstrated that RDA could be used to clone polymorphisms between two individuals and to detect viral genomes added in single copy to one genome. The applications of this method, which are myriad, are discussed here.

First, RDA may be used to search for pathogenic organisms in infected tissue (tester) that are not present in uninfected tissue from the same individual (driver) or in the pooled uninfected tissue of the patient's parents. We have demonstrated this method in reconstruction experiments but have not yet succeeded in using it to discover new pathogens in diseased tissue. We have found it necessary to extract high-molecular-weight DNA from the infected tissue, or else artifacts may arise. Three types of problems can be anticipated: when the pathogenic organism does not have a DNA phase, when the infecting organism has created a host reaction but is no longer present, and when other foreign organisms are present that are not related to the pathogenic process.

Second, RDA may be used for analysis of genetic lesions in tumors. This may proceed in either of two ways. When essentially pure tumor cells (uncontaminated by stroma) can be obtained, DNA can be extracted and used as driver against normal DNA as tester. The "target" sequences will represent restriction enzyme fragment polymorphisms that are missing in the tumor due to loss of heterozygosity or sequences that are absent in the tumor due to homozygous deletion. Such events may mark the loss of function of a tumor suppressor gene. There are three problems in applying the method in this manner. First, pairs of pure tumor cell populations and their normal counterparts are hard to come by. Second, the tumor cell source is usually a cell line, and many genetic changes may have occurred during the establishment and maintenance of the cell line. Third, loss of heterozygosity may be so prevalent that probes for these events will greatly outnumber probes for the more interesting lesions resulting from homozygous deletions. We have applied RDA in this manner in several instances and have consistently found probes that reflect loss of heterozygosity.

Alternatively, normal DNA can be taken as driver, with the tumor taken as tester. When applied this way, RDA yields probes for sequences present in tumors that are absent in normal DNA. Such sequences could arise by chromosomal rearrangements that fortuitously create small DNA restriction endonuclease fragments containing sequences not present in amplicons from normal DNA. Thus, this method has the potential to detect probes for translocations, deletions, inversions, and insertions. In addition, we have found that sequences amplified in the tumor cell can also be detected this way. If viral sequences are present in tumor cells, they will also be detected. Rarely, point mutations will create new restriction fragments that can be cloned. There are several advantages to applying RDA to tumors in the above-described manner. Material can be obtained from the patient, at all stages, by biopsy, and the contamination by normal stroma will be well tolerated. Lesions of many sorts should be identifiable and will not be confounded by loss of heterozygosity. The only disadvantage we can anticipate is that some tumor cells may have so many lesions of biological insignificance, present as a consequence of errors of DNA replication or repair, that the significant lesions cannot be found. In that event, it might be necessary to examine model animal systems.

Third, RDA can be applied to both inherited genetic disease and sporadic (or spontaneous) genetic dis-

ease. In the first case, we utilize RDA to find polymorphic markers linked to the disease. This method has been successfully tested in model systems of inherited disease in mice, carried out in collaboration with Eric Lander's group at the Massachusetts Institute of Technology. The method works because progeny with the desired phenotypes can be selected from crosses between polymorphic strains of animals, and their DNA can be used as tester (or driver) against DNA from the strain lacking the trait as driver (or tester). Similar studies with human families can be undertaken, but we have not yet done so.

In the case of sporadic disease, we can anticipate that genetic rearrangement may be responsible for a portion of spontaneous lesions. Such a situation is formally akin to genetic rearrangements in tumor cells. The affected child's DNA is used as tester, and pooled parental DNA is used as driver. We expect to be able to use RDA to isolate probes for genetic rearrangements causing a wide variety of diseases, including mental retardation, severe developmental defects, autism, and juvenile schizophrenias. Molecular approaches to such diseases were previously not generally available.

Combinatorial Chemical Libraries

R. Swanson, G. Asouline

The diversity of life is achieved by combinatorial methods: any codon can follow any codon; any amino acid the previous one. A small protein of 200 residues has 20^{200} possible sequences, a number greater than the number of atoms in the universe. From this diversity, proteins and nucleic acid sequences are selected in the course of evolution. Something like this, although not as grand in scale, can now be achieved in the laboratory by synthetic means. Oligopeptides of great diversity (in the millions or even in the billions) can be generated, either chemically or through genetic engineering (Smith, *Science 228:* 1315 [1985]; Scott and Smith, *Science 249:* 386 [1990]; Houghten et al., *Nature 354:* 84 [1991]). The structure of those few peptides selected for some characteristic (usually binding to a receptor) can then be determined. Chemists can generate many equally diverse libraries of organic molecules, but determining the structure of those few compounds that have desired properties has until now not been feasible. In collaboration with Clark Still and his colleagues at Columbia University, we have solved this problem.

Details of the method will be submitted for publication at a later time. In essence, the method entails making compounds on microsphere beads, each bead having a high density of a single compound covalently attached. This method of synthesis has been described previously by other investigators in the generation of vast peptide libraries (Furka et al., *Int. J. Pept. Protein Res. 37:* 487 [1991]; Lam et al., *Nature 354:* 82 [1991]), but whereas the identity of the compounds on a selected bead was previously determined by microsequencing, a method applicable to oligopeptides and oligonucleotides, we perform the synthesis of the compounds on the beads in such a way that the chemical identity of the compound on the bead is readily determined by "reading" the bead. Using this method, we succeeded in synthesizing a peptide library of 10^5 different molecules, selected beads bearing peptides reactive with a monoclonal antibody, and correctly decoded the beads so selected. The method is not restricted to peptides. It is applicable to the discovery of small organic molecules that bind to a given target, inhibit a given enzyme, inhibit the growth of a given microbe, or, more generally, induce a given response in cells. Other uses of the methodology will be in the study of molecular recognition.

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TRANSMEMBRANE SIGNALING

D. Bar-Sagi M. Boyer, K. Degenhardt, N. Gale, J. Hong, L. Graziadei, S. Kaplan

The control of cell proliferation by growth factors depends on an orderly transfer of information from the cell surface to the nucleus. Much of the research into growth control mechanisms in eukaryotic cells centers on the proteins encoded by the *ras* proto-oncogenes. Ras proteins are members of a large superfamily of low-molecular-weight proteins that bind guanine nucleotides. They are essential for the transduction of diverse extracellular signals that control cell growth, and abnormal activation of Ras proteins apparently contributes to the development of several types of human cancer. Our laboratory has been studying the mechanisms of signal transduction by Ras proteins. During the past few years, a number of cellular proteins involved in the control of the activity of Ras proteins have been identified. Our current studies are aimed at uncovering how these proteins interact with Ras proteins to regulate their activity. The characterization of the intermolecular interactions required for Ras activity should provide insights into the function of Ras proteins in normal cell proliferation and in oncogenic transformation.

Role of Ras Proteins in Signaling via the B-cell Antigen Receptor

L. Graziadei

B lymphocytes express antigen receptors in the form of surface immunoglobulins (sIg). Cross-linking of these receptors by antigen or anti-immunoglobulin antibodies (anti-Ig) initially induces receptor clustering (patching) followed by receptor migration to one pole of the cell (capping) and then by receptor internalization. Receptor cross-linking is the seminal event in the activation of these cells to proliferate and differentiate. We have previously found that antibody-mediated cross-linking of sIg leads to the

activation of Ras as indicated by a substantial increase in the proportion of the "active" GTP-bound form of cellular Ras molecules. This indicated that Ras can receive signals from cross-linked sIg. During the past year, we have further investigated the nature of these signals.

Cross-linking of sIg is known to activate a number of signal transduction events, including the metabolism of phosphoinositides that leads to the activation of protein kinase C (PKC). To determine whether PKC might regulate Ras activation, we have employed phorbol myristate acetate (PMA), a pharmacological activator of PKC. We have found that treatment of cells with PMA leads to a dramatic and prolonged activation of Ras. However, inhibition of PKC activity by pretreatment of cells with the PKC inhibitor Calphostin had no effect on sIg-mediated Ras activation, indicating that PKC is probably not a physiological regulator of sIg-mediated Ras activation. Cross-linking of sIg has been shown to activate protein tyrosine kinases (PTK) of the *src* family. To examine the role of PTK in sIg-mediated Ras activation, we compared sIg-mediated PTK activity (as assessed by Western blotting with anti-phosphotyrosine antibodies) with sIg-mediated Ras activation and found that the two display similar times of onset. In addition, both Ras activation and PTK stimulation are prolonged when cells are stimulated at 0°C but show rapid down-regulation at 37°C. This is consistent with the notion that Ras activation lies downstream from sIg-mediated PTK activity. To further understand the regulation of Ras activation by the sIg receptor, we are currently characterizing the activities of Ras GTPase activators and guanine nucleotide exchange factors in untreated and anti-Ig-stimulated B lymphocytes.

Differential Regulation of Ras-GAP and Neurofibromin in B Lymphocytes

M. Boyer [in collaboration with D. Gutmann and F. Collins, University of Michigan]

The activity of a Ras protein is regulated by the guanine nucleotide that is bound to the protein. The GTP-bound state is active, whereas the GDP-bound state is not. The proportion of Ras in the active state is believed to be determined principally by two reactions. An inactive GDP-bound Ras molecule is activated by the exchange of a bound GDP molecule for a GTP molecule. An active GTP-bound Ras molecule is inactivated by the intrinsic GTPase activity of the Ras protein. This inactivation reaction is greatly stimulated by Ras-GTPase-activating proteins (GAPs). To date, two GAPs have been identified: One with a molecular mass of 120 kD (Ras-GAP) and another with a molecular mass of 250 kD that is encoded by the neurofibromatosis type I gene (neurofibromin). Ras-GAP and neurofibromin are both widely expressed and have similar biochemical properties in vitro with respect to activation of Ras GTPase. We have been interested in determining the biological significance of the occurrence of these two GAPs. Immunofluorescence studies performed previously in our laboratory have demonstrated a spatial and temporal coincidence between the ligand-induced migration of sIg and Ras in mitogenically stimulated B lymphocytes. We have employed this relocalization event to analyze the involvement of Ras-GAP and neurofibromin in the Ig-mediated signaling pathway.

To study the intracellular localization of neurofibromin and Ras-GAP in B lymphocytes, we used antibodies directed against noncatalytic domain epitopes. We established the specificity of these antibodies by immunoblotting analysis as well as by immunoprecipitation from metabolically labeled B lymphocytes. By means of double-immunofluorescence labeling experiments, we found that neurofibromin accumulates under caps and patches induced by anti-Ig antibodies. In contrast, no change in the intracellular distribution of Ras-GAP was detected during the anti-Ig-induced capping. By examining the kinetics of neurofibromin redistribution, we found that the movements of neurofibromin and sIg were spatially and temporally coordinated in both the patching and the capping stages. Neurofibromin was found to co-cap with a different isotype of sIg, indicating that the redistribution of neurofibromin is a general feature of signaling by sIg. We next examined the specificity of the neurofibromin redistribution by analyzing the localization of neurofibromin during the capping of Con A receptors. Capping of Con A receptors was not accompanied by the relocalization of neurofibromin, suggesting that the redistribution of neurofibromin is specifically associated with Ig signaling. These results clearly show that neurofibromin and Ras-GAP can be differentially regulated in vivo. Furthermore, together with our

findings that Ras proteins also co-cap with sIg, our observations are consistent with the possibility that in B lymphocytes, Ras proteins interact specifically with neurofibromin to form a signaling complex. We are currently investigating the mechanisms that may regulate the assembly of this signaling complex and its potential functional significance.

The GRB2 Protein: A Component of the Ras Signaling Pathway

K. Degenhardt, J. Hong, S. Kaplan [in collaboration with J. Schlessinger, New York University]

The genetic dissection of signal transduction pathways in *Caenorhabditis elegans* has provided new insights into the components of the Ras signaling pathway. In *C. elegans*, normal vulval development requires the function of let-23, a tyrosine kinase growth factor receptor structurally similar to the EGF receptor, and let-60, a Ras homolog. Genetic screening identified sem-5, which functions downstream from the let-23 receptor and upstream of the let-60 Ras homolog. The *sem-5* gene encodes a protein that consists entirely of two SH3 domains and one SH2 domain, SH standing for src homology. The mammalian homolog of *sem-5*, GRB2, has been recently identified in the laboratory of J. Schlessinger by virtue of its ability to bind to tyrosine phosphorylated epidermal growth factor (EGF) receptor. We sought to determine whether GRB2 is a component of the Ras signaling pathway in mammalian cells. To this end, purified GRB2 and Ha-Ras proteins were microinjected into quiescent cells and their effects on DNA synthesis were examined. We found that GRB2 stimulated DNA synthesis when microinjected along with the Ha-Ras protein. In contrast, neither GRB2 nor Ha-Ras alone was ineffective. Moreover, GRB2 proteins containing mutations shown to impair *sem-5* functions were unable to induce DNA synthesis when microinjected with Ha-Ras. These results are consistent with a role for GRB2 in activating Ras.

To further examine the capacity of GRB2 to regulate mitogenic signaling by Ras, we analyzed the effects of constitutive overexpression of GRB2 on NIH-3T3 cells. NIH-3T3 cells transfected with GRB2 were morphologically indistinguishable from nontransfected cells. However, when GRB2 was introduced into NIH-3T3 cells overexpressing Ha-Ras, the transfected cells displayed transformed morphology that was clearly different from that of the parental cells. Figure 1 (b–e) shows the morphology of the parental NIH-3T3 cell line and three GRB2-overexpressing cell lines. The parental cell line resembled normal NIH-3T3 cells (Fig. 1a) in that the cells were flat, only slightly elongated, and grew as a monolayer. In contrast, the GRB2-overexpressing cells were invariably rounded, refractile, and piled on top of each other. In addition, the GRB2-over-expressing cells formed colonies in soft agar, whereas the parental cell line did not (Fig. 1f–h). These observations indicate that GRB2 contributes a positive signal toward the growth-promoting activity of Ras, thereby leading to the acquisition of the transformed phenotype. Studies are under way to determine the functional position of GRB2 in the Ras signaling pathway and the mechanisms by which GRB2 contributes to the mitogenic activity of Ras protein.

Regulation of Ras Activity by Receptor Tyrosine Kinases

N. Gale

A principal intracellular signaling pathway by which tyrosine kinases regulate cell growth involves the activation of Ras by its conversion from the GDP- to the GTP-bound state. The mechanisms by which this activation is achieved are unknown. On the basis of the structural and biochemical properties of GRB2 and the current knowledge of SH2 and SH3 domain function, a plausible pathway emerges: It is likely that the SH2 domain of GRB2 interacts with the tyrosine phosphorylated EGF receptor, whereas the SH3 domains interact with a Ras regulator. Thus, GRB2 is thought to function as a molecular adaptor linking receptor tyrosine kinases to Ras signaling. To test this hypothesis, we have analyzed the effects of GRB2 overexpression on the activation of Ras by EGF. We found that the activation of Ras by EGF is potentiated in cells overexpressing GRB2 (Fig. 2). Activation of MAP kinase by EGF, which is dependent on Ras activation, is also potentiated in cells overexpressing GRB2. These results indicate that GRB2 overexpression leads to the enhanced activation of Ras. The activity of Ras is tightly regulated by two types of proteins: GAPs that act by stimulating the GTPase activity of Ras and guanine nucleotide

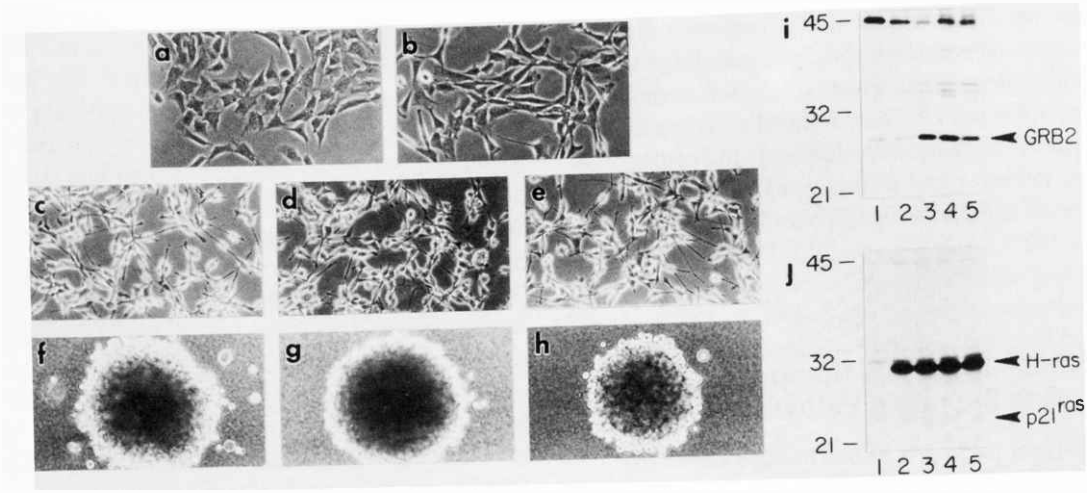

FIGURE 1 Morphologic transformation induced by constitutive overexpression of GRB2 in NIH-3T3 cells overexpressing wild-type Ha-*ras*. Phase-contrast micrographs of normal NIH-3T3 cells (*a*), NIH-3T3 cells overexpressing wild-type Ha-*ras* (*b*), and NIH-3T3 cell line GRB2 (*c*, *d*, and *e*, respectively). (*f*, *g*, *h*) Micrographs of soft agar colonies from cell lines shown in *c*, *d*, and *e*, respectively. Expression of GRB2 (*i*) and *ras* (*j*) in NIH-3T3 cells (lane *1*), NIH-3T3 cells (lane *2*) overexpressing Ha-*ras*, and NIH-3T3 cells overexpressing GRB2 (lanes *3–5*). Lysates from [^{35}S]methionine-labeled cells were immunoprecipitated with anti-GRB2 serum (*i*) or with anti-*ras* monoclonal antibody Y13-259 (*j*). For all immunoprecipitations, equal numbers of cpm were compared. Immunoprecipitates were separated on a 12.5% polyacrylamide gel followed by autoradiography at −70°C. The autoradiogram in panel *i* was exposed for 16 hr, whereas the autoradiogram in panel *j* was exposed for 48 hr to show that the levels of endogenous p21^{ras} were identical among the various cell lines. The electrophoretic mobility of the transfected wild-type Ha-*ras* is slower owing to the presence of peptide epitope at the amino terminus. Migration of molecular size standards is indicated at left in kilodaltons.

exchange factors that act by promoting the exchange of a bound GDP molecule for a GTP molecule. To determine the mechanisms by which GRB2 potentiates the EGF-induced activation of Ras, we examined the effects of GRB2 overexpression on GAP activity and on the exchange rate of guanine nucleotide on Ras. These studies revealed that the potentiating effect of GRB2 overexpression on the EGF-induced activation of Ras results from the enhancement of the rate of guanine nucleotide exchange on Ras. We also found that GRB2 immunocomplexes contain a guanine nucleotide exchange factor for Ras. In response to EGF stimulation, the GRB2 exchange factor complexes bind to the activated-EGF receptor. We conclude from these results that GRB2 can control the activation of Ras signaling by linking receptor tyrosine kinases to a guanine nucleotide exchange factor for Ras.

Future work will focus on examining the nature of the interaction between GRB2 and the Ras exchange factor and how this interaction promotes the activation of Ras.

Identification of a Guanine Nucleotide Exchange Factor for Ras

S. Kaplan, D. Bar-Sagi [in collaboration with P. Chardin, CNRS, Valbonne, France]

The regulation of guanine nucleotide exchange on Ras is a critical aspect of signaling via Ras, as only the GTP-bound form of Ras is active. Little is known about the biochemical and biological properties of guanine nucleotide exchange factors for Ras. The first exchange factor for Ras protein to be identified was the *CDC25* gene product of *Saccharomyces cerevisiae*. Genetic analysis indicated that *CDC25* plays an essential role in the activation of Ras proteins. In *Drosophila*, the protein encoded by the *son of sevenless* gene, *sos*, contains a domain that shows significant homology with the catalytic domain of *CDC25*. P. Chardin has recently cloned two human homologs of *sos*, termed hSos1 and hSos2. hSos1 and hSos2 are expressed in a wide

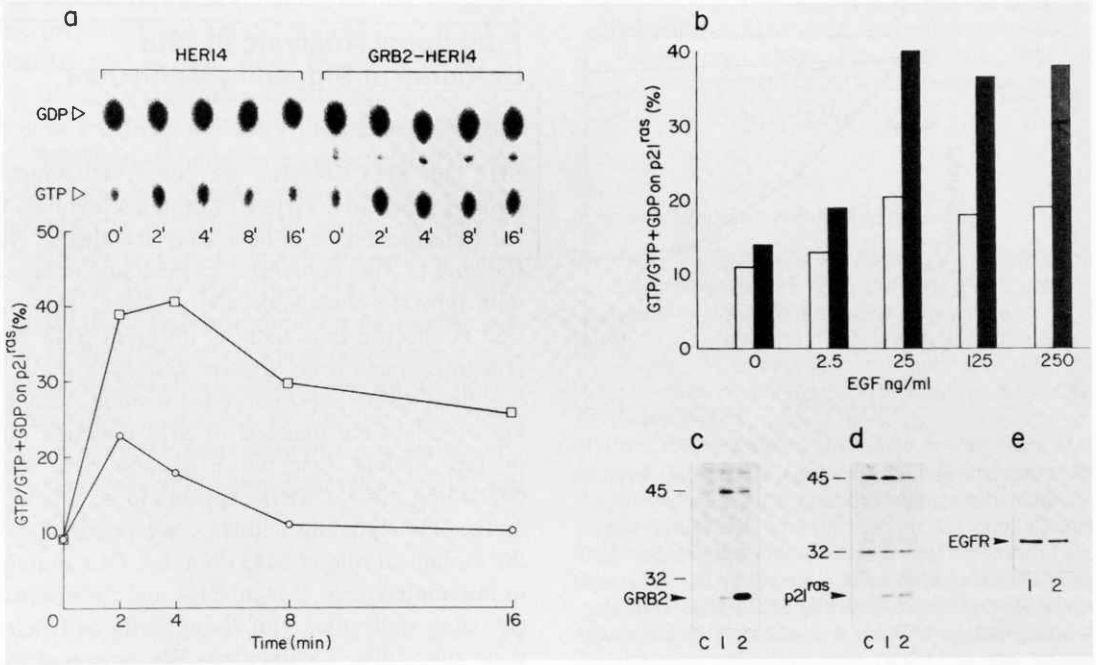

FIGURE 2 Effect of GRB2 overexpression on EGF-induced Ras activation. The cell lines used for these experiments were HER14, an NIH-3T3 cell line overexpressing the EGF receptor, and GRB2-HER14, an HER14 cell line overexpressing GRB2. (*a*) Time courses of EGF-induced increase in GTP-bound to Ras in HER14 cells (*open circle*) and in GRB2-HER14 cells (*open box*). The $p21^{ras}$ was immunoprecipitated with the *ras*-specific monoclonal antibody Y13-259 from cells labeled with ^{32}P-orthophosphate that had been stimulated for the indicated times with 250 ng/ml EGF. Nucleotides were eluted, separated by thin-layer chromatography, and quantitated using a phosphorimager. The thin-layer chromatogram corresponding to the quantitative data is shown in the inset. The positions of GDP and GTP standards are indicated. (*b*) EGF concentration dependence of the nucleotides bound to Ras in HER14 cells (*open bars*) and in GRB2-HER14 cells (*closed bars*). ^{32}P-labeled cells were incubated with the indicated concentrations of EGF for 2 min. At the end of the incubation, Ras was immunoprecipitated, and the amounts of nucleotides bound to Ras were quantitated as in *a*. (*c–e*) Expression of GRB2, Ras, and EGF receptor in HER14 cells and in GRB2-HER14 cells. (*c*) GRB2 was immunoprecipitated using anti-GRB2 serum. (*d*) Ras was immunoprecipitated using anti-$p21^{ras}$ serum. (*e*) Western blot of EGF receptor. Protein (50 μg) from each cell line was electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose sheet. The nitrocellulose sheet was incubated with anti-EGF receptor sera, washed, and incubated with ^{125}I-labeled protein A. Immunoprecipitations were carried out as described from lysates of cells labeled with [^{35}S]methionine. Immunoprecipitates were analyzed on 12.5% polyacrylamide gels. Migration of molecular size standards is indicated at left in kilodaltons. (Lane *1*) HER14 cells; (lane *2*) HER14 cells overexpressing GRB2; (lane *C*) control immunoprecipitation carried out in HER14 cells by using antibodies that were blocked by preincubation with an excess of purified antigen. Anti-GRB2 serum was incubated with 5 μg of purified GRB2 fusion protein (panel *c*), and anti-Ras serum was incubated with 5 μg of purified human Ha-*ras* protein (panel *d*).

range of tissue types, consistent with a role as positive regulators of the ubiquitously expressed Ras genes. We have carried out a set of biochemical experiments aimed at demonstrating that hSos proteins are in fact guanine nucleotide exchange factors for Ras. A purified fragment of hSos1 corresponding to the *CDC25*-related domain was able to induce significant stimulation of guanine nucleotide exchange on recombinant Ras proteins. This activity appeared to have a rather strict specificity toward Ras, since no effect could be detected on the rate of guanine nucleotide exchange on the Ras-related protein RalA. Moreover, mammalian cells overexpressing the full-length hSos1 have increased guanine nucleotide exchange activity (Fig. 3). These results establish that hSos1 is a guanine nucleotide exchange factor for Ras.

The identification of the Ras exchange factor should enable us to obtain a much more complete picture of how Ras is regulated. One of our immediate goals will be to find out how the Ras exchange factor is activated.

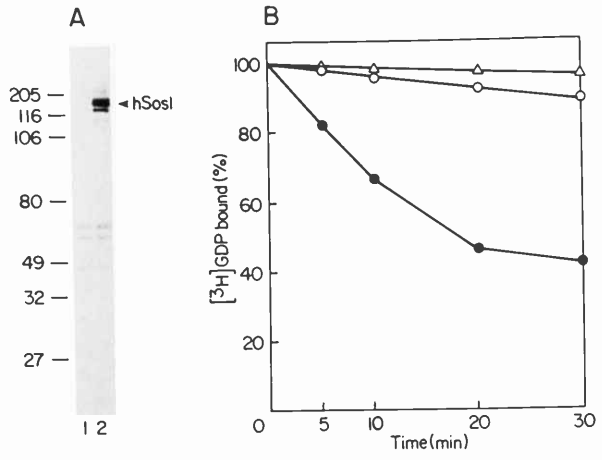

FIGURE 3 Acceleration of exchange of GDP by extracts prepared from cells overexpressing the full-length hSos1. (*A*) Immunoblots of cell lysates prepared from human kidney 293 cells transfected with a control expression vector (lane *1*) or with an expression vector encoding the full-length hSos1 (lane *2*). Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose strip which was then probed with anti-hSos1 antibodies. The immunoreactive bands were visualized using the ECL detection system. (*B*) Effects of cell extracts on the kinetics of GDP dissociation. [^{3}H]GDP-bound Ha-*ras* (5 pmoles) was incubated at 37°C with 500 μl of extracts (6 mg/ml) from human kidney 239 cells (*open circles*), from human kidney 293 cells overexpressing the hSos1 protein (*closed circles*), or with buffer (*open triangles*), in the presence of 2 mM unlabeled GTP. At the indicated times, Ha-*ras* was immunoprecipitated, and the amount of [^{3}H]GDP associated with the immunocomplexes was determined. Results are plotted as percentages relative to the values obtained at 0 min. Each datapoint represents the average of three independent determinations.

Functional Analysis of SH3 Domains of Signaling Molecules

D. Bar-Sagi

SH2 and SH3 domains are conserved noncatalytic regions found in a variety of proteins involved in signal transduction. It is now well established that the function of SH2 domains is to mediate the interaction with tyrosine phosphorylated proteins. This interaction is specific inasmuch as different SH2 domains recognize specifically a short sequence motif flanking the tyrosine phosphorylated residue. Very little is known about the function of SH3 domains. In view of our finding that the SH2 and SH3 domain-containing protein GRB2 appears to be a component of the Ras signaling pathway, we opted to examine the biological role of SH3 domains. Our strategy was to microinject recombinant SH2 and SH3 domains of signaling molecules into living cells and determine their subcellular localization. We reasoned that the functional specificity of different signaling molecules might be reflected in their differential cellular localization. The dependence of a specific localization pattern of a given signaling molecule on the SH3 domain(s) could then be used as an assay for the function of these domains. The SH2 and SH3 domain-containing proteins that we chose to use were PLCγ and GRB2. Using immunofluorescence microscopy, we found that a truncated protein composed of the SH2 and SH3 domains of PLCγ was localized to the actin cytoskeleton. We found the same localization pattern when only the SH3 domain of PLCγ was injected, whereas injected protein composed of only

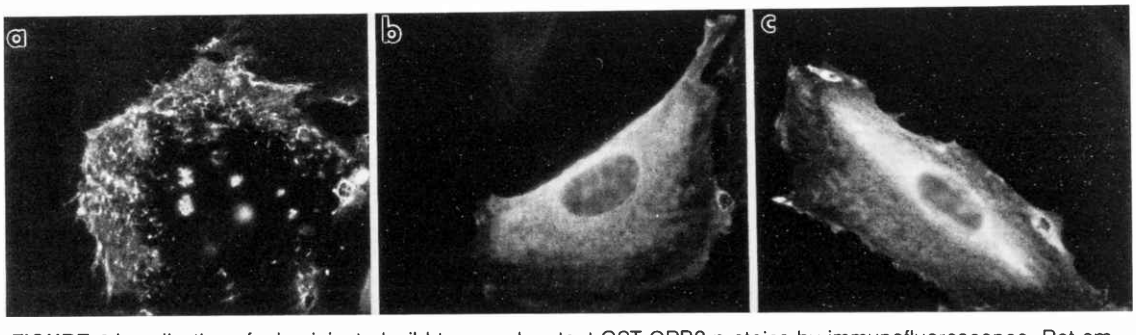

FIGURE 4 Localization of microinjected wild-type and mutant GST-GRB2 proteins by immunofluorescence. Rat embryo fibroblast 52 cells were microinjected with either wild-type GRB2 protein (*a*) or with mutant GRB2 proteins containing point mutations at the amino-terminal SH3 domain (*b*) or the carboxy-terminal SH3 domain (*c*). All proteins were microinjected at a concentration of 1 mg/ml. Four hours after injection, cells were fixed, permeabilized, and stained with anti-GST rabbit serum followed by rhodamine-conjugated goat anti-rabbit Ig. Magnification, 1240x.

the SH2 domain of PLCγ exhibited diffuse cytoplasmic distribution. These results indicate that it is the SH3 domain that is responsible for the targeting of PLCγ to the actin cytoskeleton. Microinjected GRB2 protein was localized primarily to membrane ruffles Fig. 4). SH2 loss-of-function mutants of GRB2 exhibited the same localization pattern as the wild-type protein, whereas SH3 loss-of-function mutants of GRB2 failed to localize to membrane ruffles. These results are consistent with the idea that the SH3 domains of GRB2 are responsible for its specific cellular localization. Together, these observations suggest that SH3 domains are responsible for the targeting of signaling molecules to specific subcellular locations. We will be examining this concept specifically in the context of the linkage between GRB2 and Ras signaling.

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NUCLEAR SIGNAL TRANSDUCTION

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Our major focus continues to be the mechanisms by which extracellular signals are communicated to the nucleus to control the proliferation and development of eukaryotic cells. We view this as a two-part problem. First, we need to understand the simple mechanics of the process. What are the individual molecular steps by which signals are relayed from the cell surface to nuclear targets? Second, we need to understand where the biological specificity in these signals is encoded. How do cells know what signal they have received and respond appropriately?

Our general approach to these questions has revolved around studies of the c-*fos* proto-oncogene. This gene is rapidly activated at the transcriptional level by a variety of extracellular stimuli, including growth and differentiation factors, hormones, and neurotransmitters. Induction of c-*fos* transcription occurs within seconds or minutes of exposure to these signaling molecules and does not require the cell to synthesize new proteins. Thus, activation of the c-*fos* gene is a primary response to the signal transduction pathways activated by these factors, and our working hypothesis is that the c-*fos* gene itself is a physical target for these signals.

Consequently, our general strategy has been to identify the sequences that mediate the response of the c-*fos* gene to specific signal transduction pathways. Using these sequences as reagents, we then identify and characterize the cellular proteins that interact with them, and we eventually use these proteins as reagents to identify the next proteins up the signaling chain. As described below, we have

made varying degrees of progress working our way up the different signal transduction pathways that activate c-*fos* transcription. Of particular importance are recent experiments in which we have been able to recapitulate at least one such signal transduction pathway in a cell-free system.

In addition to the mechanics of signal transduction to the nucleus, we have a strong interest in understanding the specificity of this process. How does a given cell distinguish among different signals that trigger overlapping signal transduction pathways? How do different cells manage to respond in unique ways to the same signal? We know, for example, that we can generate c-*fos* promoter derivatives that respond to only a subset of the signals that normally activate c-*fos* transcription, indicating that different signals use different routes to the nucleus. In some cases—cAMP, for example—this is due to the use of completely distinct *cis*-acting regulatory elements and *trans*-acting factors. In other cases, different signals target distinct nuclear proteins that interact with a common sequence element. In this way, it is possible for different signals to target distinct subsets of genes. A second insight into the specificity of signaling comes from our recent discovery that homeodomain proteins may participate in the regulation of gene transcription by extracellular signals. Since homeodomain proteins play a role in establishing the specificity of cell identity in embryonic development, we suspect that the interaction of these proteins with the proteins responsible for signal-activated transcription may be an important aspect of how the specificity of signaling is achieved. We are pursuing these studies at structural and functional levels and have begun a project to test this hypothesis in the context of *Drosophila* embryonic development.

Distinct Protein Complexes at the SRE Mediate Growth Factor Induction of c-*fos* Transcription

M. Gilman, R. Graham, H. Sadowski

The serum response element (SRE) is required for the response of the c-*fos* gene to at least two distinct signal transduction pathways, one dependent on protein kinase C (PKC) and one or more independent of PKC. In addition, the SRE is target for the rapid repression of c-*fos* transcription that follows induction. Given the complexity of SRE function, it is not surprising that the SRE binds several different cellular DNA-binding proteins. One of these proteins, SRF, appears to be required for all activities of the SRE. Our working hypothesis is that SRF works by recruiting an array of distinct proteins to the SRE and that it is these accessory proteins that carry signaling information to the SRE. One such accessory protein is a 62-kD protein termed ternary complex factor (TCF), recently identified as a member of the ETS family. TCF specifically recognizes the SRF-SRE complex, probably contacting both SRF and the SRE. Mutant SREs that bind SRF but fail to support formation of the SRF-TCF complex lose response to one of the two signal transduction pathways that target the SRE. They fail to respond to the PKC pathway, although they respond at wild-type levels to PKC-independent signals.

In contrast, we find that the response to PKC-independent signals requires the cooperation of SRF with a different protein. This protein, termed SIF, binds to a sequence 25 bp upstream of the SRE, and in contrast to SRF and TCF, its DNA-binding activity is induced by growth factors (see below). In the context of a ternary complex mutant that responds only to PKC-independent signals, we find that this residual response can be abolished by mutations that affect the binding of either SRF or SIF, suggesting that these two proteins cooperate to mediate the response to these signals. Particularly noteworthy is the observation that a double mutant that fails to bind TCF or SIF but still binds SRF loses nearly all response to signals, strongly suggesting that SRF alone is not sufficient for response to any signals and that it functions exclusively via recruitment of pathway-specific accessory proteins.

Regulation of c-*fos* Transcription by Receptor Tyrosine Kinases

H. Sadowski

Signal transduction to the nucleus by growth factor receptors with protein-tyrosine kinase activity is complex. The initial event in activation of these types of receptors is the cross-linking of receptors by ligand, resulting in the rapid and concerted *trans*-phosphorylation of the receptors on multiple tyrosine residues. Phosphorylation on tyrosine is absolutely required for receptor signaling. The next step in receptor signaling is the recruitment of a series of

cytoplasmic and membrane-associated signaling proteins to the receptor where they may or may not be phosphorylated on tyrosine. Many of these proteins associate with phosphotyrosine residues on the receptor via protein domains termed SH2 (src homology region-2) domains. Each of these SH2-domain-containing proteins is thought to elicit an independent intracellular signal that is propagated to downstream targets. Thus, activation of growth factor receptors elicits a complex array of intracellular signaling events.

Much of our research has focused on how transcription of the c-*fos* gene is activated by polypeptide growth factors such as platelet-derived growth factor (PDGF/c-*sis*) and epidermal growth factor (EGF), whose cell surface receptors possess ligand-inducible tyrosine kinase activity. Growth factor receptor signaling to the c-*fos* gene involves both PKC-dependent and -independent pathways. As described above, PKC-dependent signals act through a ternary complex composed of SRF and TCF, whereas PKC-independent signals appear to act through SRF in conjunction with a different accessory factor, termed SIF, which binds to a sequence approximately 20 bp upstream of SRF. Unlike SRF and TCF, SIF exists in a latent form in unstimulated cells that does not bind DNA. SIF DNA-binding activity is induced within minutes of exposure of cells to growth factors, without a requirement for protein synthesis. These observations suggest that SIF is physically modified in response to growth factor signal transduction. SIF activity is induced by several growth factors that act via receptors with intrinsic protein-tyrosine kinase activity, including PDGF (both the AA and BB homodimers), EGF, and insulin, depending on the cell type. It is not induced by PMA, an activator of protein kinase C (PKC), nor is SIF activation affected by depletion of PKC, suggesting that SIF activation is PKC-independent.

Using A431 cells as a model system, we have examined the mechanism of SIF activation by EGF. Activation of SIF by EGF does not involve the well-characterized signal transduction pathways utilizing PKC, calcium, or cAMP, but it clearly requires tyrosine kinase activity as activation is inhibited by cell-permeable tyrosine kinase inhibitors. SIF activation is extremely rapid at 37°C, detectable within 20 seconds of EGF addition, peaking within 4 minutes, and persisting for at least 60 minutes. Three distinct mobility-shift complexes appear in nuclear extracts, whereas only the two more rapidly migrating complexes appear in the cytoplasm. When activations are performed at 0°C, where internalization of receptors and nuclear transport of proteins is inhibited, SIF activation is still readily apparent within 30 seconds with apparent accumulation over time of the two faster-migrating complexes in the cytoplasmic fraction relative to the nuclear fraction. Together, these data suggest that latent SIF may exist in the cytoplasm in cells where it is very rapidly activated by a receptor-proximal event (kinetics just behind receptor tyrosine phosphorylation), after which the DNA-binding-competent SIF is then translocated to the nucleus. UV cross-linking studies with nuclear extracts identified at least three polypeptides in the 90–94-kD range that were site-specifically cross-linked and EGF-inducible. Similarly sized [^{35}S]methionine-labeled polypeptides were detected using site-specific DNA-affinity precipitations of extracts from EGF-stimulated cells. Phosphorylation is required for SIF DNA-binding activity, as treatment of extracts with nonspecific phosphatase abolishes all three mobility-shift complexes. Furthermore, at least one of the complexes requires tyrosine phosphorylation, as its formation is abolished when DNA-affinity-purified SIF is treated with a T-cell protein phosphotyrosine phosphatase.

Since the activation of SIF by EGF in A431 cells is quite robust and a receptor-proximal event, we have used fractions from unstimulated A431 cells to develop a cell-free assay for SIF activation. In this assay, we observe time- and ligand-dependent activation of SIF, with a requirement for detergent-treated A431 membranes, cytoplasm (from virtually any cell), ATP, Mg, and Mn. Antibody depletion experiments indicate that activation is absolutely dependent on the EGF receptor. Activation is blocked by antibody to phosphotyrosine, by recombinant SH2 domains, and by free phosphotyrosine, indicating that at least one phosphotyrosine-SH2 domain interaction is required for SIF activation. We are using this cell-free signal transduction system to further characterize the mechanism of SIF activation.

Purification and Characterization of the SRE-binding Protein p62DBF

S. Natesan

Previous work by Bill Ryan in our laboratory identified a novel 62-kD SRE-binding protein, termed

$p62^{DBF}$, which binds directly to the 5′ side of the SRE. Our recent data suggest that $p62^{DBF}$ is antigenically indistinguishable from the mammalian transcription factor YY1. YY1 is a ubiquitously expressed zinc finger protein that functions as a transcriptional repressor or activator, depending on its context. A survey of the mouse c-*fos* promoter revealed the presence of three binding sites for this protein, located in the −310, −255, and −55 regions of the c-*fos* promoter. Both recombinant YY1 and affinity-purified HeLa $p62^{DBF}$ bind to these sequences.

Previous data from our laboratory indicated that $p62^{DBF}$ may be a DNA-bending protein. Circular permutation and phasing analyses clearly show that both YY1 and $p62^{DBF}$ bend the DNA at all three sites in the c-*fos* promoter; at the −310 site within the SRE, the angle of the bend is 72°. Because DNA bending may play an important role in transcriptional regulation by organizing the tertiary structure of promoter DNA, we have tested the importance of the YY1-induced bend at −55 in the c-*fos* promoter. Mutations in this YY1-binding site increase the basal rate of c-*fos* promoter activity by approximately fivefold, suggesting that this site normally represses transcription. Surprisingly, however, reversing the orientation of the YY1 site increases promoter activity an additional fivefold, suggesting that in the opposite orientation, this site activates transcription. We believe that this unusual orientation dependence may reflect bending by YY1. Moreover, these effects were not observed when the CRE element immediately upstream of the YY1-binding site was absent. This observation suggests that YY1 has no intrinsic activity at this site, but instead acts to interfere with or facilitate—depending on its orientation—the interaction of CRE-binding proteins with the basal transcription machinery.

An examination of the interaction of YY1 at the SRE suggested that YY1 enhances the rate of binding of SRF to this site. Furthermore, cotransfection of a YY1 expressor plasmid with a reporter containing a single SRE leads to the activation of transcription, suggesting that YY1 may also enhance SRF binding in vivo. However, we are unable to demonstrate co-occupancy of the SRE by SRF and YY1. It is possible that YY1-mediated bending of the SRE lowers the free energy required for binding of SRF to the SRE, a process that requires only transient occupancy of SRE by both proteins. Thus, the function of YY1 in the context of SRE may be to alter the local DNA structure to facilitate rapid assembly of SRF and associated factors on SRE.

Cloning and Characterization of SRE-ZBP, a Novel SRE-binding Protein

R. Attar

As described above, the SRE is a target for multiple signals that activate and repress c-*fos* transcription. To understand the complex mechanisms by which the SRE regulates the c-*fos* transcription, we have attempted to identify and clone previously unidentified SRE-binding proteins. We screened a HeLa cell cDNA expression library for phage-expressing proteins that specifically bound an SRE oligonucleotide. We isolated a phage that encoded a LacZ fusion protein that specifically bound this oligonucleotide but not a mutant site. The fusion protein binds specifically to the SRE in both a Southwestern blot and a mobility-shift assay. Analysis of the partial DNA sequence of this clone revealed that it was a previously unidentified gene that belongs to the family of zinc-finger-containing proteins related to the *Drosophila Krüppel* gene.

The clone contains seven tandem repeats in the carboxyl terminus that match the zinc finger consensus for this gene family. This gene is expressed in a variety of human cell lines at a very low level. Expression is induced by serum in HeLa cells with delayed kinetics relative to c-*fos*. Methylation interference experiments showed that SRE-ZBP interacts with the 3′ side of the SRE. Immunofluorescence assays on cells transfected with an SRE-ZBP expression vector indicate that the protein is localized to the nucleus.

Before investigating the role of this protein in the regulation of the c-*fos* transcription, it was necessary to isolate the full-length cDNA. We used the RACE (*R*apid *A*mplification of *c*DNA *E*nds) procedure to extend the existing sequence 208 nucleotides in the 5′ direction. To confirm that these new sequences correspond to the SRE-ZBP gene, we utilized a probe derived from the 5′ end of the original cDNA to isolated a 17-kb human genomic fragment carrying the SRE-ZBP gene. Within this fragment, we found con-

ntiguous sequences corresponding to the RACE clones and the original cDNA, indicating that the new cDNA sequence represented the authentic 5′ end of the SRE-ZBP mRNA.

Primer-extension experiments suggested that the mRNA begins 120 bp upstream of the putative initiation codon for SRE-ZBP. This ATG codon is in-frame with the original cDNA; upstream of this ATG, there are stop codons in all three reading frames. At positions −31 and −20 of the putative initiation of transcription are two perfect TATA boxes. Together, these data suggest that we have located the 5′ end of the SRE-ZBP mRNA.

The full-length SRE-ZBP protein expressed in *E. coli* runs as a 70-kD protein, in agreement with earlier observations that antibodies raised against recombinant SRE-ZBP recognized a 70-kD protein in human cells. Interestingly, in contrast to amino-terminally truncated forms of SRE-ZBP, the full-length protein does not bind to the SRE. This observation raises the possibility that SRE-SBP contains a domain that inhibits the DNA-binding activity of the zinc finger region.

Inactivation of this inhibitory domain may require covalent modification of the protein or association with another cellular factor. These issues, together with ongoing efforts to ascertain the function of SRE-ZBP in c-*fos* regulation, will be the focus of our future studies.

MADS Box–Homeodomain Interactions and the Specificity of Signal Transduction to the Nucleus

D. Grueneberg

As described earlier, SRF is required for the response of the c-*fos* gene to growth factor signals, but it appears to function by recruiting an array of accessory proteins with distinct activities. In this regard, SRF resembles the yeast protein Mcm1, both of which are members of the MADS box family of transcription factors. Like SRF, Mcm1 is also involved in the regulation of genes by extracellular signals, the mating pheromones, and it functions in cooperation with accessory proteins. We exploited these similarities to devise a genetic selection in yeast for human cDNAs encoding proteins that cooperate with Mcm1 to activate a cell-type-specific pheromone-responsive reporter gene. We isolated a cDNA encoding a novel human homeodomain protein, Phox1, with a homeodomain most closely related to the *paired* class, initially identified in *Drosophila*.

Our initial characterization of Phox1 indicated that in addition to interacting with Mcm1 to activate the yeast reporter gene, Phox1 is also capable of interacting with the human protein SRF. The effect of this interaction in vitro is the enhancement of binding of SRF to the SRE. This effect is mainly kinetic. Phox1 enhances both the rate of association and the rate of dissociation of the SRF-SRE complex, acting essentially as an exchange factor to ensure that SRF reaches equilibrium on the SRE rapidly. This activity resides within the Phox1 homeodomain and is shared with other homeodomains of the *paired* class but not with more distantly related homeodomains. The enhancement activity does not require the DNA-binding activity of the homeodomain, suggesting that direct interaction between SRF and the Phox1 homeodomain is required.

We have recently devised an assay to study the interaction between Phox1 and SRF in vivo. In HeLa cells, the homeodomain of Phox1 transcriptionally activates a reporter gene containing an SRE. Our results suggest that Phox1 activation of the reporter requires an interaction with SRF, because a reporter carrying an SRE mutant that binds Phox1 but not SRF is not activated by Phox1. Mutational analysis of Phox1 suggests that activation of the reporter, and presumably SRF-Phox1 interaction, requires both DNA binding and direct Phox1-SRF interactions. Interestingly, mutations that affect the DNA-binding specificity of the homeodomain also affect the efficiency of complex formation as measured in the HeLa cell assay. Moreover, different homeodomains also display different activities in this assay. The related *Drosophila* homeodomain protein Paired has about 40% of the activity of Phox1, whereas the unrelated *Drosophila* homeodomain protein Deformed is not active. We speculate that SRF will interact with different homeodomain proteins on different binding sites, and we are in the process of identifying such binding sites. Interactions between homeodomain proteins and MADS box proteins may account for how homeodomain proteins function in early development to specify transcriptional responses to inductive signals.

Mutagenesis of the Homeodomain-SRF Interface

K. Simon

Homeodomain proteins, first characterized in the fruit fly *Drosophila melanogaster*, have key regulatory functions in development. Mutations in their genes cause changes in cell identity. Homeodomain proteins bind DNA and act as transcription factors, although their specificity of action cannot be predicted by their DNA-binding activity alone, leading to the suggestion that homeodomain function requires cooperation with other proteins. As described above, we have cloned a novel human homeodomain protein, Phox1, that interacts with the yeast MADS box protein Mcm1 to restore transcriptional activity in a reporter gene. Phox1 also interacts with the human MADS box protein SRF to enhance its binding to the SRE in the c-*fos* promoter. As in yeast, Phox1 is also able to activate an SRF-dependent reporter gene in mammalian cells.

To understand the interaction between Phox1 and SRF, we have begun to construct and test a series of Phox1 mutants. Mutations in the DNA recognition helix of the Phox1 homeodomain abolish the ability of Phox1 to cooperate with SRF to activate a reporter gene, suggesting that DNA-binding activity of the homeodomain is required for this activity. Moreover, mutations that affect the DNA-binding specificity of the homeodomain also influence this activity, suggesting that the primary recognition specificity of the homeodomain is also important. Initial mutagenesis suggests that homeodomain residues oriented away from the DNA are also required for interaction with SRF, suggesting a role for direct homeodomain-SRF contact. Thus, we are currently mutagenizing the Phox1 homeodomain to target residues predicted to reside on the solvent-exposed surface of the homeodomain, where they can potentially make contact with SRF. We are using three assays to measure Phox1-SRF interactions: in vitro DNA binding, a reporter gene assay in HeLa cells, and a similar assay in yeast. Our goal is to identify the key amino acid side chains in the Phox1 homeodomain that contact SRF and, having identified those, to identify next the corresponding contact sites in SRF.

We believe that homeodomain/MADS box protein interactions could impart specificity of action to homeodomain proteins in general and that these interactions are one way in which a developing organism can initiate cell-type-specific transcriptional activation that is responsive to external signaling pathways.

Isolation and Characterization of MADS Box Proteins in *Drosophila*

A. Ryan, R. Attar

Development of the fruit fly *Drosophila melanogaster* provides an attractive system to test specific predictions associated with our studies of SRF-homeodomain interactions. Homeodomain proteins were first discovered in *Drosophila*, and their functions in embryonic development are relatively well understood. Indeed, it is principally from biological studies of homeodomain function in *Drosophila* embryos that it has become evident that homeodomain proteins most likely cooperate with as yet unidentified protein partners. Our data on SRF-homeodomain interactions suggest that proteins of the SRF (or MADS box) family are good candidates for homeodomain partners. Consequently, we are isolating SRF-related genes from *Drosophila*.

Members of the MADS box family include SRF and a family of related human proteins, the yeast protein Mcm1, and a group of plant genes with homeotic functions in flower development. The MADS box itself is a moderately conserved block of about 60 amino acids that forms part of a larger DNA-binding and protein-dimerization domain. This domain is also implicated in the interaction of SRF and Mcm1 with various accessory proteins, including proteins of the homeodomain family.

We are using a combination of low-stringency hybridization and polymerase chain reaction (PCR) to screen *Drosophila* genomic and cDNA libraries for MADS box genes. Candidate genes will be sequenced and mapped to polytene chromosomes to determine if they correspond to any known developmental genes. The expression patterns of the genes during embryonic development and in the adult fly will be examined by Northern analysis and in situ hybridization. The availability of these clones should allow us to test directly whether these proteins do function as homeodomain partners during development and, additionally, to address the role of SRF-like proteins in the well-characterized signal transduction pathways active during fly development.

Characterization of MADS Box Structure and Function in Yeast

C. Alexandre

It is clear that several striking parallels exist between growth factor signal transduction pathways in animal cells and the pheromone response pathway in yeast. These parallels include the use of G proteins and related protein kinase cascades and the organization of signal-responsive regulatory elements in target genes. In particular, cell-type-specific pheromone-responsive genes in yeast are under the control of a regulatory gene, *MCM1*, that encodes a protein closely related to SRF; these proteins have overlapping DNA-binding specificities and participate in similar types of protein-protein interactions. We are exploiting these similarities in several ways: by producing human proteins in yeast to study their activities directly, by producing hybrid proteins derived primarily from yeast proteins but carrying small domains from their human counterparts, and, on occasion, by directly studying the yeast proteins in the hope of learning something that will be relevant to our understanding of the human proteins.

To study the activity of human proteins produced in yeast, we have established yeast strains carrying reporter genes under the control of the human c-*fos* SRE. In these strains, we are producing various human proteins, including SRF, Elk-1 (the ternary complex factor that interacts with SRF), and components of the MAP kinase cascade. Our goal here is to reconstitute a functional mammalian signal transduction pathway in yeast, where simple genetic tools can be used to study the structure and function of these proteins. Our use of hybrid proteins is focused principally on studying the interaction between SRF and Elk-1, which we believe is analogous to the interaction between the yeast proteins Mcm1 and α1. Interaction of SRF and Elk-1 appears to require amino acids within the dimerization domain of SRF and a short Elk-1 domain, termed the B box. We find a sequence within the α1 protein that is closely related to the B box, suggesting that this region of α1 interacts with Mcm1. Indeed, a four-amino-acid insertion within the B box homology of α1 inactivates the protein. Thus, we have constructed two hybrid genes, an Mcm1 derivative carrying the dimerization domain of SRF and an α1 derivative carrying the B box of Elk-1. Simple genetic assays can tell us whether these hybrid proteins can interact productively in yeast. If so, then we will use these assays to probe genetically the organization of this protein-protein interface. We hope to extend this analysis eventually to include the Phox1 homeodomain, allowing us to study a putative quaternary complex of DNA, SRF, Elk-1, and Phox1, which may be analogous to the yeast Mcm1/α1/Ste12 complex.

Control of c-*fos* Expression by Calcium in T Cells

G. Lee

Calcium ions function as second messengers in a variety of physiological processes, including proliferation, muscle contraction, and neuronal signaling. Its role as an effector is accomplished primarily through transient fluxes in concentration. Several calcium-binding proteins are thought to mediate the action of calcium signals. Rapid changes in cytoplasmic calcium concentration can occur via membrane channels or by release from internal stores. In many cases, transient increases in calcium occur in tandem with activation of PKC, and the combination of these signals is often synergistic. A clear case of the synergy of calcium and PKC occurs during the activation of T cells by antigen. Indeed, treatment of T cells with calcium ionophores and PKC activators can fully mimic antigen activation, although each treatment alone is ineffective. This same synergy is also evident at the level of c-*fos* expression, suggesting that by understanding the synergistic control of c-*fos* transcription by calcium and PKC, we may understand in a general way how these important signal transduction pathways interact.

Our studies of c-*fos* induction in T cells suggest that synergy arises from two distinct calcium-mediated effects on c-*fos* expression. One signal affects promoter function and enhances the initiation of c-*fos* transcription in cooperation with PKC activators. A second signal affects the elongation of c-*fos* transcripts, by transiently alleviating a block to elongation within the first intron of the c-*fos* gene. Several lines of evidence support this conclusion. The major evidence comes from nuclear run-on assays that measure the rate of transcription of discrete portions of the c-*fos* gene. Treatment of T cells with the PKC activator PMA results in enhanced transcription of the 5′ end of the c-*fos* gene, but these tran-

scripts do not proceed much beyond the end of the first exon and consequently little mature c-*fos* mRNA is made. In the presence of both PMA and a calcium ionophore, two changes are observed. First, there is enhanced transcription of the first exon, suggesting an increase in the rate of transcript initiation at the c-*fos* promoter. Second, a much higher fraction of initiated transcripts continue beyond the first exon and through the entire gene. Thus, through a combination of enhanced initiation and more efficient elongation of the primary c-*fos* transcript, a synergistic effect on the level of mature c-*fos* mRNA is observed. These two effects of calcium are independent of one another, because in combination with agents that elevate intracellular cAMP, the elongation effect is seen, although there is no synergy at the level of initiation. This observation is confirmed by transfection assays of the c-*fos* promoter function in T cells, which measure only initiation effects and do not recapitulate the effect of calcium on elongation. We observe that PMA and calcium cooperatively enhance c-*fos* promoter activity, whereas calcium has no effect on induction of the c-*fos* promoter by cAMP. Importantly, calcium also fails to affect the function of the transcription factor CREB, thought to be a major focus for calcium action in neuronal cells.

Our current work is focused on the mechanisms through which calcium cooperates with PKC. Preliminary data suggest that this cooperation is not simply due to enhanced activation of PKC in the presence of elevated calcium, because we see little enhancement of protein phosphorylation in the presence of PMA and ionomycin. Transfection of mutant c-*fos* promoters suggests that the SRE is required for the synergistic effect of calcium and PKC on c-*fos* promoter activity, but surprisingly, it is not sufficient. This observation suggests that synergy requires the cooperation of the SRE with an additional element in the c-*fos* promoter. Our data clearly rule out the SIE and CRE elements for this role, and we are currently focused on an AP1-related site located immediately adjacent to the SRE.

It will be difficult to establish the mechanism through which calcium regulates elongation of c-*fos* transcripts, because this phenomenon is not faithfully recapitulated by transiently transfected c-*fos* genes. This observation suggests that appropriate regulation of elongation may require an integrated transcription unit or the assembly of the gene into chromatin. Nevertheless, using the nuclear run-on assay, we can distinguish between two possible models for how elongation is modulated. We envision two general possibilities. First, there may be a discrete block to transcript elongation that is removed in the presence of calcium. Second, the c-*fos* promoter could load a poorly processive form of RNA polymerase II in the absence of calcium, and calcium regulates the association of an elongation factor that enhances the efficiency of elongation. By performing nuclear run-on analysis using a series of small probes that span the entire c-*fos* transcription unit, we find that there is a sharp drop in transcription between the first exon and first intron and no change in transcription beyond this point. In the presence of calcium, transcription of all downstream regions increases coordinately. This observation argues in favor of a discrete and relatively complete block, rather than a gradual diminution of RNA polymerase density, as predicted by the processivity model.

Linking Growth Factors to Cell Cycle Control: Transcriptional Regulation of Cyclin D Genes

K.-A. Won [in collaboration with Yue Xiong and David Beach, Cold Spring Harbor Laboratory]

How the initial intracellular signaling events triggered by growth factors are linked to the regulatory machinery that controls cell cycle progression in mammalian cells is still poorly understood. The transition from the G_0 to the G_1 phase of the cell cycle involves quantitative and qualitative changes in the expression of many genes and their products. Within minutes after growth factor or serum stimulation and independently of protein synthesis, a set of genes, termed the immediate-early genes, becomes transcriptionally active. Yet, not all signals that activate the immediate-early genes cause cells to enter or leave the cell cycle. To understand how cells are able to discriminate among such signals, we are studying the regulation of a family of genes whose expression may be critical to the control of cell cycle progression: the G_1 cyclins.

Yue Xiong and David Beach here at Cold Spring Harbor Laboratory have cloned a family of human cyclin genes termed D-type cyclins. One of these genes, cyclin D1, appears to correspond to the oncogene *bcl-1*, whose chromosomal locus is amplified or translocated in many tumors. We have examined

the expression of the cyclin D1 and D3 genes in primary human fibroblasts undergoing the G_0/G_1 transition. We find that transcription of these genes is induced by mitogenic growth factors, with increased mRNA levels appearing 2–4 hours after stimulation and peak levels appearing just prior to the onset of S phase. Cyclin D expression is induced by all signals that are mitogenic for these cultures, consistent with the idea that cyclin D expression is required for the G_1/S transition. But the genes are also activated by signals such as phorbol esters that are not mitogenic in these cells, suggesting that cyclin D expression is not sufficient for entering S phase. In addition, induction of cyclin D gene expression by mitogens is lost in senescent cells that reside permanently in the G_0 stage of the cell cycle. Our data suggest that the role of cyclin D may not be in the regulation of the G_1/S transition but rather in the entry into and exit from the quiescent G_0 state.

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STRUCTURE, FUNCTION, AND REGULATION OF PROTEIN TYROSINE PHOSPHATASES

N.K. Tonks S.M. Brady-Kalnay, R.L. Del Vecchio, A.J. Flint, A.J. Garton, P.M. Guida, Jr., K.R. LaMontagne, C.A. Ostman, A.A. Samatar, H. Sun, Q. Yang

The phosphorylation of tyrosyl residues in proteins is a key component of the control of many fundamental physiological processes. Our laboratory is particularly interested in the role of tyrosine phosphorylation in transducing an extracellular signal into an intracellular response, such as proliferation or differentiation. Most laboratories have focused their studies on the protein tyrosine kinases. However, phosphorylation is a reversible, dynamic process in which the net level of phosphate observed in a target substrate reflects not only the activity of the kinases that phosphorylate it, but also the competing action of protein phosphatases that catalyze the dephosphorylation reaction. We direct our attention to the expanding family of protein tyrosine phosphatases (PTPases) that like the kinases, comprise both transmembrane, receptor-linked forms and nontransmembrane cytosolic species (Fig. 1). The structures of the PTPases indicate important roles in the control of processes such as cell adhesion, cytoskeletal function, and the cell cycle. Clearly, their characterization will generate a more sophisticated understanding of the precise physiological roles of tyrosine phosphorylation.

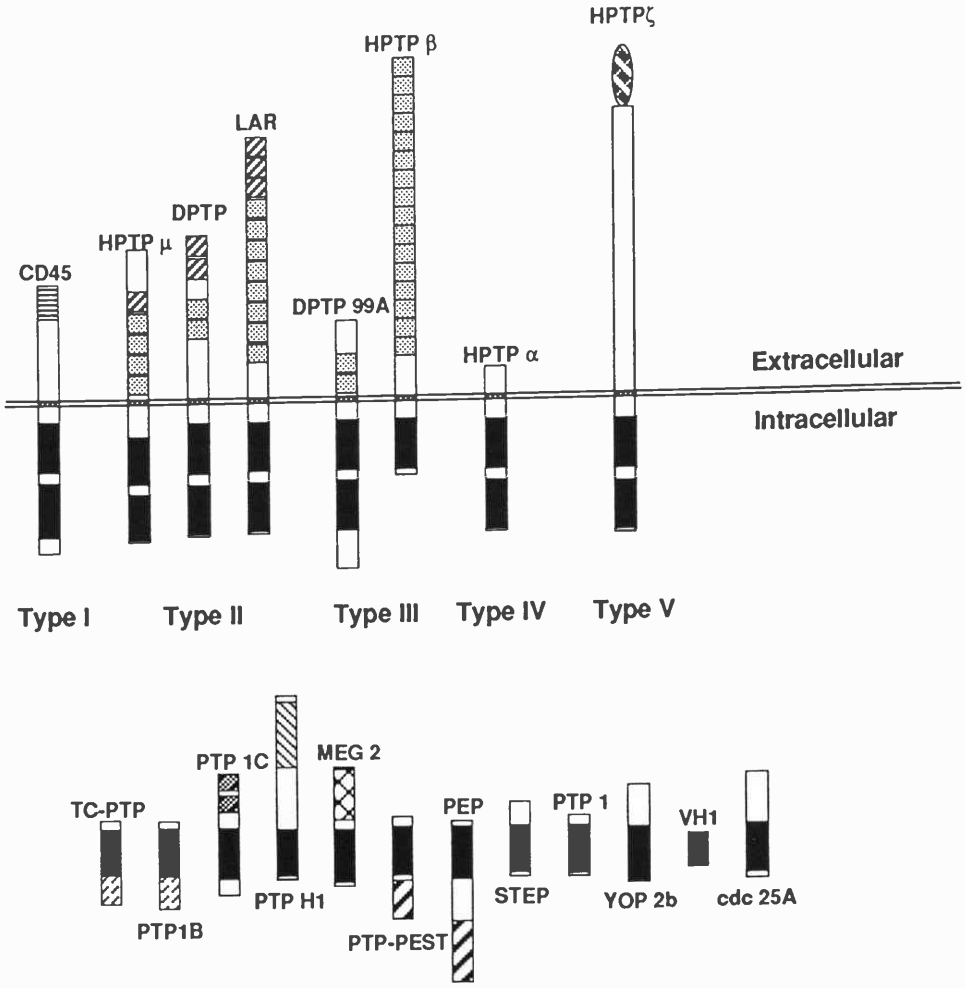

FIGURE 1 Members of the PTPase family. This figure summarizes work from many groups. The conserved catalytic domains are shown in black. The PTPs can be categorized as transmembrane, receptor-like, or nontransmembrane molecules. At the present time, the receptor-like species can be subdivided into five types based on the structure of their extracellular segments. Type I represents the CD45 family, multiple isoforms of which arise from differential splicing of a primary mRNA transcript of a single gene; three exons encoding sequences at the extreme amino terminus (*horizontal lines*) are differentially expressed. Type II contains immunoglobulin-like (*diagonal lines*) and tandem fibronectin-type-III-like repeat domains (*stippled*); this category includes LAR (leukocyte common antigen-related), DLAR, DPTP, and HPTPμ. Type III bears multiple fibronectin-type-III-like repeats. Some type III isoforms such as HPTPβ have only one internal PTP domain. Type IV isoforms such as α and ε have small glycosylated extracellular segments. Type V possesses an amino-terminal motif that has homology with carbonic anhydrase. Multiple nontransmembrane forms have also been identified. Many of the nonreceptor PTPs bear noncatalytic segments that are structurally related to other well-characterized proteins. The position and relative size of these noncatlytic domains are shown as boxes containing distinct symbols; noncatalytic regions that have similar sequences are designated with identical patterns. The noncatalytic segments that have been identified include two SH2 domains in PTP1C and band 4.1 homology domains in PTPH1, an apparent lipid-binding domain in MEG2, and segments containing PEST sequences in PEP and PTP-PEST. In TC-PTP and PTP1B, the carboxy-terminal noncatalytic segments appear to play a role in modulating activity and controlling subcellular localization. PTP1, STEP, and Yop2b have noncatalytic sequences that are apparently unrelated to sequences in the databases. The protein from vaccinia virus, VH1, is much smaller than the other PTPs and presumably encodes only essential sequences within the catalytic domain. VH1 differs from the other members of the family in that it displays dual specificity, dephosphorylating PSer as well as PTyr. (Reproduced from *Adv. Second Messenger Phosphoprotein Res. 28:* [203].)

The Receptor-like Protein Tyrosine Phosphatase, PTPμ, Mediates Cell-Cell Aggregation

S.M. Brady-Kalnay, N.K. Tonks

PTPμ is characterized by the following arrangement of structural motifs (Gebbink et al., *FEBS Lett. 290:* 123 [1991]. Its extracellular segment contains one immunoglobulin (Ig)-like and four fibronectin (FN)-type-III-like domains and thus displays structural similarity to members of the Ig superfamily of cell adhesion molecules that includes NCAM, the neural cell adhesion molecule. There is a single transmembrane domain and a large intracellular segment containing two PTPase domains preceded by a juxtamembrane segment that is 70 residues longer than the equivalent segment in other receptor-like PTPases. We have observed that this juxtamembrane region of PTPμ displays homology with the intracellular domain of members of the cadherin family of cell adhesion molecules. Such homology is unique among both the receptor-like PTPases and members of the Ig superfamily.

NCAM mediates homophilic adhesion; i.e., NCAM on one cell binds to NCAM on an adjacent cell. To investigate whether PTPμ could also serve in cell-cell adhesion, we have followed the strategy of expressing the protein in normally nonadhesive cells to test whether this induces aggregation. To pursue this goal, we have generated recombinant baculoviruses to express various forms of PTPμ in Sf9 cells. We have demonstrated that expression of full-length PTPμ, or mutants bearing an intact extracellular segment, whether in the presence or absence of the PTPase domains, induced aggregation (see Fig. 2). Expression of the catalytic segment of PTPμ in Sf9 cells as a soluble protein did not induce aggregation, suggesting that increased PTPase activity alone was not sufficient for this response. Furthermore, expression of a chimeric molecule containing the extracellular and transmembrane domains of the EGF receptor and the intracellular segment of PTPμ was similarly ineffective. Thus, overexpression of a nonspecific transmembrane protein was insufficient to induce aggregation. The degree of aggregation of Sf9 cells induced by PTPμ expression is very similar to that observed following expression of other well-characterized adhesion molecules in various model systems.

The binding reaction has also been reconstituted in vitro. We have shown that full-length PTPμ linked to fluorescent beads bound specifically to surfaces coated with the extracellular segment of PTPμ, purified following expression as a fusion protein in bacteria. Binding is thus independent of metal ions and glycosylation. These observations suggest that PTPμ-mediated aggregation is induced through homophilic binding; i.e., the "ligand" for this receptor-like PTPase is a molecule of the same enzyme expressed on an adjacent cell. This view is supported by the observation that when fluorescently labeled uninfected cells are mixed with unlabeled cells expressing full-length PTPμ, labeled cells are excluded from the aggregates.

As far as we can tell, at least in this system, aggregation, i.e., ligand binding to the extracellular segment, had no detectable direct effect on the activity of the intracellular PTPase domains. Nevertheless, if this holds true for cells in which PTPμ is normally expressed, it is possible that such an interaction could serve a tethering role, controlling the activity of the PTPase indirectly by restricting its spatial distribution on the membrane and thus restricting the spectrum of substrates with which it may interact. The homology between the juxtamembrane segment of PTPμ and the intracellular domain of the cadherins may be informative in this regard. The intracellular domain of the cadherins is the most highly conserved segment among members of this family (90% identity) and is essential for adhesion. Through interactions with molecules called catenins, this domain directs association with the actin cytoskeleton. The cadherins are localized at adherens-type cell junctions. Cell junctions are areas of rapid phosphotyrosine turnover and locations at which kinases such as *src* and pp125^{FAK} are concentrated in normal and transformed cells. In fact, changes in tyrosine phosphorylation may be involved in controlling the structural integrity of these junctions. It is possible that the cadherin-related juxtamembrane segment of PTPμ may associate with the cytoskeleton through interaction with a catenin-like molecule at points of cytoskeleton-membrane association such as at intercellular junctions. PTPμ offers a unique, potentially direct link between cell-cell adhesion and the triggering of signal transduction pathways. The homology of PTPμ with the cadherins, NCAM-like molecules, and PTPases predicts an interesting role in cell-cell communication, and continuing studies of PTPμ should yield important new insights into the control of signal transduction processes.

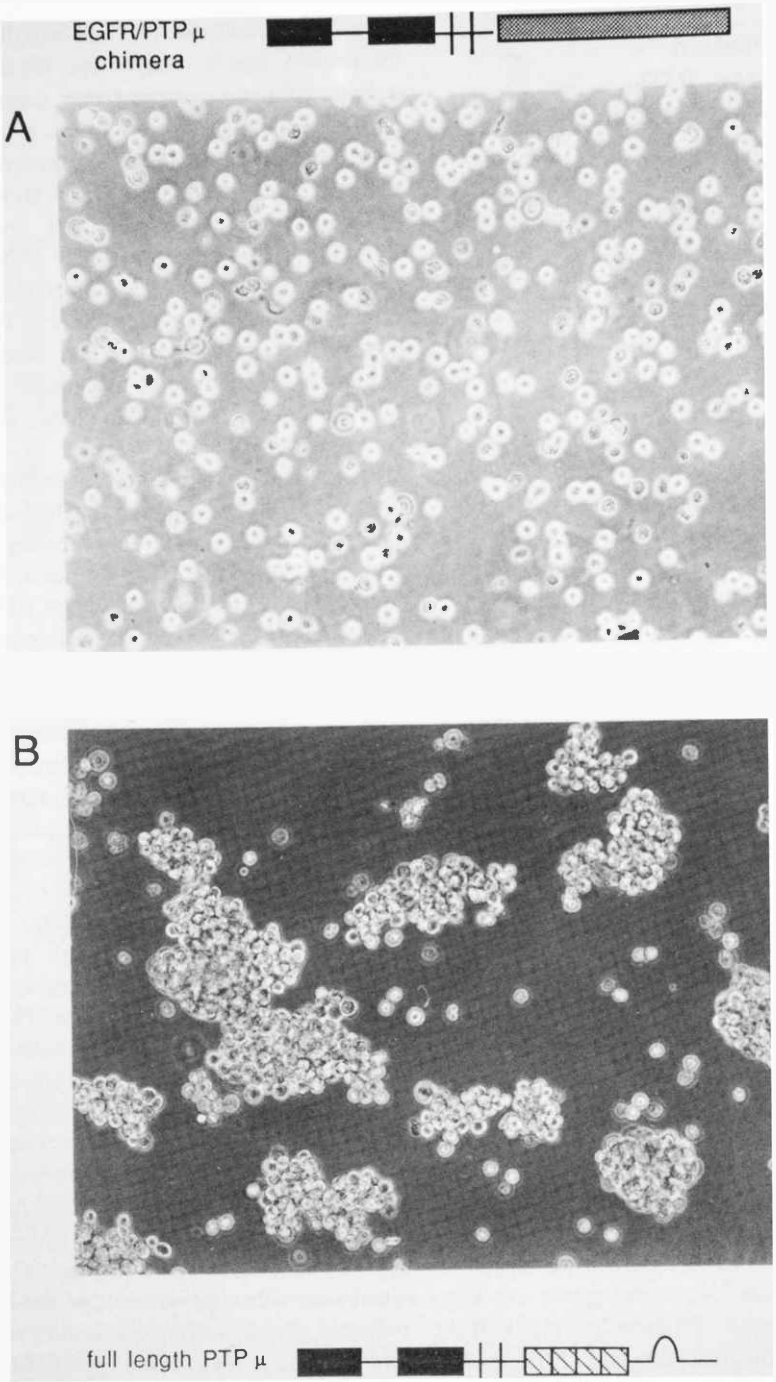

FIGURE 2 Aggregation of Sf9 cells following expression of various forms of PTPμ from recombinant baculoviruses. The picture shows phase-contrast micrographs of infected Sf9 cells after aggregation under low shear conditions for 1 hr. The cells in panel *A* express a chimeric molecule comprising the extracellular and transmembrane segments of the EGF receptor fused to the intracellular PTPase domains of PTPμ. These cells did not form aggregates. The same observation was made for uninfected Sf9 cells or cells expressing the phosphatase domains of PTPμ as a soluble protein. In contrast, panel *B*, cells expressing the full-length form of PTPμ form large aggregates.

The Growth Factor Inducible Immediate-Early Gene 3CH134 Encodes a Protein Tyrosine Phosphatase

H. Sun, N.K. Tonks [in collaboration with C.H. Charles and L.F. Lau, University of Illinois]

Serum growth factors stimulate cell proliferation through interactions with their specific cell surface receptors, activating the intrinsic receptor protein tyrosine kinases (PTKs). This leads to signal transduction events that include the reversible phosphorylation of a number of cellular substrates on tyrosyl residues. Some of these signals are transmitted to the nucleus, where the activation of a set of immediate-early genes occurs. These immediate-early genes encode a diverse array of regulatory proteins, including transcription factors and cytokines. Their expression has been hypothesized to mediate the biological effects of the stimulatory growth factor.

3CH134 is an immediate-early gene, activated rapidly and transiently in quiescent fibroblasts treated with serum growth factors (Charles et al., *Oncogene 7:* 187 [1992]). 3CH134 is also expressed during liver regeneration soon after partial hepatectomy, further suggesting a link between its expression and the control of cell growth. Its transcription in fibroblasts is detected within minutes of growth factor addition, reaches a peak level by 10–20 minutes, and is repressed by 1 hour. The 3CH134 mRNA has a very short half-life and accumulates for only 1–2 hours during the G_0/G_1 transition. Likewise, the 3CH134 protein is transiently synthesized and has a half-life of 40 minutes. Of particular interest is the observation that the sequence of the 3CH134 protein contains the motif [I/V]HCXAGXXR[S/T]G that defines the members of the PTPase family. We have purified a bacterially expressed form of the 3CH134-encoded protein and demonstrated that it possesses intrinsic PTPase activity in vitro.

The identity of the physiological substrates of 3CH134 remains to be established. In light of the rapid and transient appearance of 3CH134 mRNA and protein following stimulation of quiescent cells with serum or growth factors, a role in the regulation of early steps in signal transduction from growth factor receptor PTKs is possible. At least two sites of action would be consistent with such a role: the receptor PTKs or the downstream serine/threonine kinases that are themselves regulated by reversible tyrosine phosphorylation. As an example of the former category, we assayed PTyr-BIRK, the recombinant catalytic domain of the insulin receptor, which has been used as a substrate for PTPases in vitro. No significant dephosphorylation by purified 3CH134 was detected. Candidate substrates in the latter category include the MAP kinases. A diverse array of hormones and growth factors elicit a rapid and transient activation of MAP kinases as an essential step in their signaling pathways. Maximal activation of MAP kinases requires phosphorylation of both a tyrosyl residue and a threonyl residue residue in the protein; dephosphorylation of either severely attenuates activity. We have demonstrated that 3CH134 dephosphorylates PTyr-$p42^{mapk}$ in vitro 15-fold more rapidly than PTyr-RCM lysozyme and 200-fold more rapidly than the phosphorylated synthetic peptide EDNDYINASL, both of which are standard substrates used in the characterization of members of the PTPase family. The possibility that, among other potential physiological substrates, 3CH134 acts on members of the MAP kinase family in vivo clearly merits further attention. Much of the study of growth factor signaling cascades thus far has focused on the kinases. Our observations regarding 3CH134 point to a new class of PTPase whose activity is regulated at a transcriptional level as well as through protein turnover, suggesting that 3CH134 may play a role in attenuating the cellular response to stimulated growth factor receptors. Thus, these signal transduction pathways may be fine-tuned at the level of both phosphorylation and dephosphorylation of key enzyme constituents.

A Prokaryotic Protein Tyrosine Phosphatase

H. Sun, N.K. Tonks [in collaboration with M. Potts and P.J. Kennelly, Virginia Tech]

Interest in PTPases from bacterial sources has been piqued by the observation of Guan and Dixon (Ann Arbor, Michigan) that the YopH gene product, an essential virulence determinant of the pathogenic bacterium *Yersinia*, the causative agent of the Plague or Black Death, is a member of this enzyme family. They have gone on to show that the PTPase activity of the YopH protein is essential for virulence. The enzyme, which is encoded on a 70-kb megaplasmid, exerts its effects by dephosphorylating target proteins in the infected cell.

Nostoc commune is a heterocystous cyanobacterium, colonies of which grow in depressions formed in limestone rock in Karst scenery. *N. commune* has the ability to fix nitrogen in specialized differentiated cells termed heterocysts. During differentiation, these cells undergo structural and biochemical modifications to provide the reducing environment required for nitrogen fixation. We have identified a chromosomally encoded gene, *iphP*, from *N. commune*, which contains the [I/V]HCXAGXXR[S/T]G motif that defines members of the PTPase family. We have expressed the protein in *Escherichia coli* and purified it to homogeneity. It displays intrinsic PTPase activity toward standard substrates used in assays in vitro (e.g., RCM lysozyme). In addition, *iphP* dephosphorylated phosphoseryl residues in caseine phosphorylated by cAMP-dependent protein kinase, indicating that it may be regarded as a dual specificity phosphatase. When cell lysates of *N. commune* were probed on an immunoblot with an anti-phosphotyrosine antibody, a protein of 85 kD was detected. The signal in the immunoblot was competed out by free phosphotyrosine, but not phosphoserine or phosphothreonine, supporting the idea that p85 contains phosphotyrosyl residues. Interestingly, this immunoreactive band is detected in cells grown in the presence of combined nitrogen but not in the nitrogen-deficient media that induce heterocyst differentiation.

These data suggest a role for protein tyrosine phosphorylation and thus, potentially, the PTPase activity of *iphP* in the control of cellular function in this cyanobacterium. *iphP* in fact represents the first PTPase of genuine, unambiguous prokaryotic ancestry to be identified. Our observations raise fundamental questions as to the origin and function of tyrosine phosphorylation. Thus, it is possible that tyrosine phosphorylation may have arisen much earlier in evolution than previously anticipated, i.e., before the emergence of the eukaryotes as a distinct group.

Regulation of PTPase Activity

A.J. Flint, K.R. LaMontagne, A.A. Samatar, P.M. Guida, Jr., N.K. Tonks

The activity of members of the PTPase family may be regulated at several levels. In the case of the receptor-like enzymes, there is obviously the potential for modulation of activity by ligand binding to the extracellular segment. In addition, it appears that many of the members of this family will be responsive to regulation by reversible phosphorylation, intracellular targeting, and association with other proteins. We are currently studying these matters with particular emphasis on PTP1B and CD45. This year, considerable progress has been made in characterizing the phosphorylation of PTP1B in vivo. (Martijn Gebbink [Netherlands Cancer Institute] has also contributed to these studies.)

PTP1B is a nontransmembrane PTPase, the first member of the family to be isolated in homogeneous form. Its cDNA predicts a protein of 435 amino acids, of which the carboxy-terminal 114 residues have been implicated in controlling both the localization and function of the enzyme. Neel's group (Beth Israel Hospital, Boston) has shown that the extreme carboxy-terminal 35 amino acids, which are of a highly hydrophobic nature, are both necessary and sufficient for targeting the enzymes of the cytoplasmic face of membranes of the endoplasmic reticulum. Upon inspection of the sequence of the preceding 80 amino acids, which are predominantly hydrophilic, we noted several potential sites for phosphorylation by serine/threonine kinases. We have now confirmed that in HeLa cells, PTP1B is phosphorylated on seryl residues in vivo.

We have observed increased phosphorylation of PTP1B that coincides with the G_2/M-phase transition of the eukaryotic cell cycle. Arrest of cells in mitosis with nocodazole is associated with a decrease of 30% in the specific activity of PTP1B in vitro toward substrates such as RCM lysozyme. We have therefore characterized this process in detail. The phosphorylation of PTP1B in mitotic cells is accompanied by a pronounced retardation in its electrophoretic mobility. We utilized site-directed mutagenesis and polymerase chain reaction (PCR) to synthesize templates in which each seryl residue in the regulatory segment was individually mutated to alanine. The nocodazole-induced shift in electrophoretic mobility was then examined following expression of the various mutants in 293 cells. Mutation of a single serine, Ser-352, was sufficient to abolish the mobility shift. In two-dimensional tryptic maps of ^{32}P-labeled PTP1B from mitotic cells, two phosphopeptides were resolved. One of these comigrates with the peptide generated following phosphorylation of PTP1B on Ser-386 in vitro by the mitotic protein serine/threonine kinase $p34^{cdc2}$:cyclin B. We presume that the second

represents Ser-352. In addition to the changes in mitosis, stimulation of HeLa cells with the phorbol ester TPA also enhances phosphorylation of PTP1B, predominantly on Ser-378, which is the site labeled by protein kinase C (PKC) in vitro. Thus, the effect of TPA is mediated directly by PKC.

In vivo, PTP1B is therefore a point of convergence for the action of at least three distinct protein serine/threonine kinases as summarized in Figure 3. PKC labels Ser-378, which is a major site of phosphorylation in PTP1B from asynchronously growing cells. Mitosis is accompanied by a decrease in the phosphorylation of this site and an increase in the phosphorylation of two distinct sites, Ser-386, which is phosphorylated by $p34^{cdc2}$ in vitro and in vivo, and Ser-352. The kinase responsible for phosphorylation of Ser-352 has not yet been identified. These observations are important because they illustrate both a novel interplay between serine/threonine and tyrosine phosphorylation, as well as a potential novel tier of control of the level of cellular phosphorylation.

Characterization of Novel PTPases

R.L. Del Vecchio, A.J. Garton, C.A. Ostman, Q. Yang, N.K. Tonks

PTPX1 and PTPX10

We have been looking into *Xenopus laevis* as a model system in which to investigate how PTPases may function in cellular signaling. Degenerate primers, corresponding to conserved residues within the catalytic domain of PTPases, were used in a PCR-based protocol to isolate cDNAs representing 14 distinct phosphatase domains from *X. laevis*. Two of these cDNAs, designated X1 and X10, were chosen for further study and full-length clones were obtained. Full-length X1 encodes a protein of 738 amino acids with a predicted molecular mass of 78 kD (PTPX1); full-length X10 encodes a 652-amino-acid protein with a predicted molecular mass of 70 kD (PTPX10). Both proteins belong to the non-transmembrane family of PTPases. PTPX1 and PTPX10 share segments of 97% identity in both the carboxy-terminal catalytic domains and the amino-terminal portions. In addition, PTPX1 contains an 86-amino-acid insert between the amino- and carboxy-terminal segments of the protein. PCR analysis of genomic DNA suggests that these two PTPases are the products of two separate genes. The amino-terminal halves of both PTPX1 and PTPX10 are 28% identical to bovine cellular retinaldehyde-binding protein and 23% identical to sec14, a yeast phosphatidylinositol transferase. This similarity may indicate the potential for regulation of PTPX1 and PTPX10 by a lipid molecule. Sequence comparison and Northern blot analysis indicate that PTPX10 is likely to be the *Xenopus* homolog of the previously described human PTPMEG-2. PTPX1, on the other hand, is a highly related, yet distinct, PTPase.

We have expressed PTPX1 and PTPX10 in Sf9 cells using recombinant baculovirus, and we are currently analyzing their kinetic properties. Within the unique insert in PTPX1 are two threonine-proline motifs that are consensus sites for phosphorylation by cdc2 and MAPK. The presence of these potential phosphorylation sites may indicate that although they are structurally similar, PTPX1 and PTPX10 may be differentially regulated. PTPX1 can be phosphorylated by $p42^{mapk}$ in vitro, and the effects of this phosphorylation on phosphatase activity are currently being assessed. Using a polyclonal antibody specific for PTPX1, we are currently carrying out immunoprecipitation studies to determine whether the activity and phosphorylation state of the phosphatase are altered during the cell cycle.

PTP-PEST

We amplified PTPase-related cDNA from a template of total RNA isolated from human skeletal muscle. PTP-PEST was detected by this procedure. Full-length cDNA clones were isolated from HeLa cell cDNA libraries by screening with the PCR fragment. The cDNA predicts a protein of 780 amino acids lacking an obvious signal or transmembrane sequence, suggesting it is a nonreceptor-type enzyme. The PTPase domain is in the amino-terminal portion of the molecule. The carboxy-terminal segment is rich in proline, glutamic acid/aspartic acid, serine, and threonine residues, possessing features of PEST motifs. These motifs are found in proteins with a very short intracellular half-life, suggesting that PTP-PEST may turn over rapidly in vivo. Intrinsic activity has been demonstrated in vitro against a variety of phosphotyrosine-containing proteins, including the cytoplasmic PTK domain of the insulin receptor. As anticipated, the enzyme appears to be specific for phosphotyrosyl residues. PTP-PEST mRNA is broad-

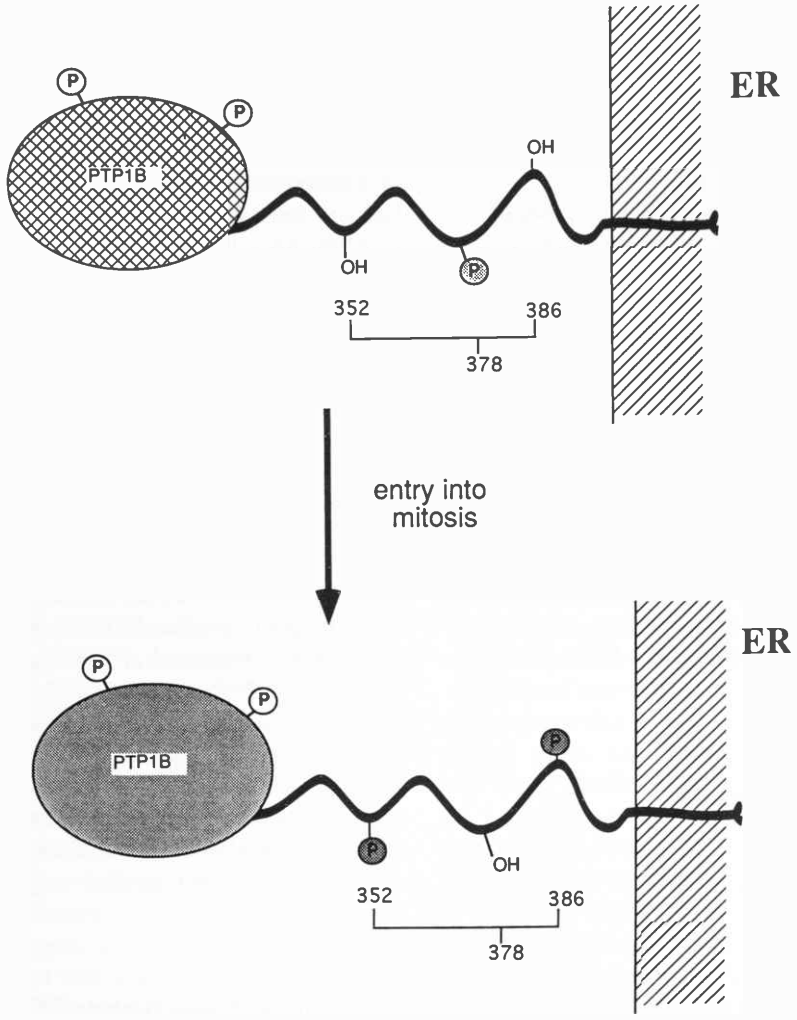

FIGURE 3 Schematic depiction of the phosphorylation of PTP1B. PTP1B is drawn with a globular catalytic domain connected by a hydrophilic spacer of 80 amino acids to its hydrophobic endoplasmic reticulum (ER) anchoring sequence. The three major sites of phosphorylation are Ser-352, Ser-378, and Ser-386. Two unidentified minor phosphopeptide species observed in the two-dimensional maps are represented by the phosphates on the globular domain. The majority of the phosphorylation of PTP1B in unsynchronized HeLa cells occurs on Ser-378. The phosphorylation state of this site is substantially increased in response to TPA treatment of the cells. However, in cells arrested in mitosis with nocodazole, phosphate is apparently lost from this site, whereas two new major phosphopeptides are detected. These sites of phosphorylation correspond to Ser-386, which is the primary site of $p34^{cdc2}$ phosphorylation in vitro, and to Ser-352, which is required for the altered electrophoretic mobility of PTP1B isolated from mitotic cells. The kinase responsible for the phosphorylation of Ser-352 has not been identified but appears not to be the $p34^{cdc2}$:cyclin B complex. (Reproduced from Flint et al., *EMBO J. 12(5)*, in press [1993].)

ly distributed in a variety of cell lines. Stimulation of rhabdomyosarcoma A204 cells, a transformed muscle cell line, with insulin led to a fourfold increase in mRNA levels within 36 hours. The possibility that PTP-PEST may play a role in signaling responses to insulin is currently being investigated.

PTPH3

We have isolated a partial cDNA clone, again from HeLa cells, for this receptor-like PTPase. Like HPTPβ isolated by Saito and co-workers (Dana Farber Cancer Center, Boston), the intracellular segment of PTPH3 comprises a single PTPase domain. We have confirmed that this possesses intrinsic phosphatase activity. The clone also encodes a transmembrane segment and an incomplete extracellular domain comprising eight repeated fibronectin type III motifs (there are 16 such motifs in HPTPβ). These motifs appear to be involved in protein:protein interactions and have been observed in a variety of receptors. We are in the process of obtaining full-length clones and characterizing this enzyme.

PTPH1

This is a nontransmembrane PTPase of 913 residues, characterized by the presence of an amino-terminal domain of 320 residues that has homology with the cytoskeleton-associated proteins: band 4.1, ezrin, and talin. This domain defines a family of proteins that are targeted to interfaces between the plasma membrane and the cytoskeleton. For example, band 4.1, which promotes the association of actin and spectrin in erythrocytes, interacts through this domain with the transmembrane protein glycophorin. We propose that PTPH1 may be similarly restricted in its intracellular localization, and we are currently trying to identify the proteins with which it may interact.

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CELL BIOLOGY OF THE NUCLEUS

D.L. Spector S. Huang, S.C. Henderson, R. O'Keefe, L.F. Jiménez-García, R. Derby, S. Landon

Studies in our laboratory are focused on the structural and functional organization of the mammalian cell nucleus. Our research program evolves around two specific areas of study: (1) elucidating the organization of specific DNA sequences (genes and chromosomal regions) in the interphase nucleus and relating this organization to function and (2) understanding the organization of factors associated with pre-mRNA splicing and the RNA substrates with which these factors interact. The microscopy core facility has been used extensively during the past year, and numerous collaborations are under way with the excellent technical expertise of Robert Derby.

Nonrandom Localization of DNA Sequences

S.C. Henderson, D.L. Spector

Fluorescence in situ hybridization was used to determine if DNA sequences occupy specific positions within human diploid interphase nuclei and if these positions change during differentiation. In myoblasts (Fig. 1a), chromosome-specific α-satellite DNA sequences appear to be randomly arranged with respect to each other, although they preferentially localize to the nuclear periphery or the nucleolar surface. However, we found that as myoblasts become confluent and begin to differentiate, the nuclei become more elliptical in shape and centromeric sequences begin to align in parallel between neighboring cells. Upon fusion of the cells into a myotube, the sister nuclei (i.e., nuclei sharing the same cytoplasm, not necessarily daughter nuclei of a common parent) align within the myotube and homologous centromeres align with the axis of alignment parallel to the long axis of the myotube (Fig. 1b,c). A similar arrangement was observed in sections of human striated muscle. In addition to α-satellite DNA sequences aligning parallel to the long axis of the myotube, particular α-satellites appeared to occupy specific positions within each nucleus. In human male myotubes hybridized in situ with X-chromosome-specific α-satellite DNA, the X centromere localized at the nuclear periphery and in a similar quadrant in many of the myotube nuclei (Fig. 1d,e). A similar phenomenon was observed for several other single-copy DNA sequences.

Our finding on the organization of DNA sequences in the interphase nucleus supports the hypothesis that as cells terminally differentiate, the organization of interphase chromatin becomes progressively more "fixed" in place. However, this restricted order does not necessarily preclude some chromosomal movement within confined domains of the nucleus upon differentiation. The hypothesis for the purpose of this rearrangement is that as cells differentiate, DNA sequences to be expressed in the terminal state are placed in an optimal nuclear environment (i.e., juxtaposed with specific protein factors and/or other DNA sequences) in order to enhance (or facilitate) gene expression. Different nuclear events may require that the chromatin structure is arranged in different ways such that specific sequences are in defined places at particular times. Furthermore, various tissue types may have specific patterns of organization of DNA sequences. By identifying patterns of DNA organization that are characteristic for particular cell types, we will be able to construct a nuclear map of the interphase position of genes. These data may be extremely useful for prenatal diagnosis and/or biopsy evaluation whereby it may be possible to identify changes in the three-dimensional spatial localization of a gene that may later manifest itself in the development of a pathological condition.

Effect of RNA Polymerase II Transcription on the Localization of Splicing Factors

S. Landon, D.L. Spector

Pre-mRNA splicing factors are localized in a speckled distribution within mammalian cell nuclei. We

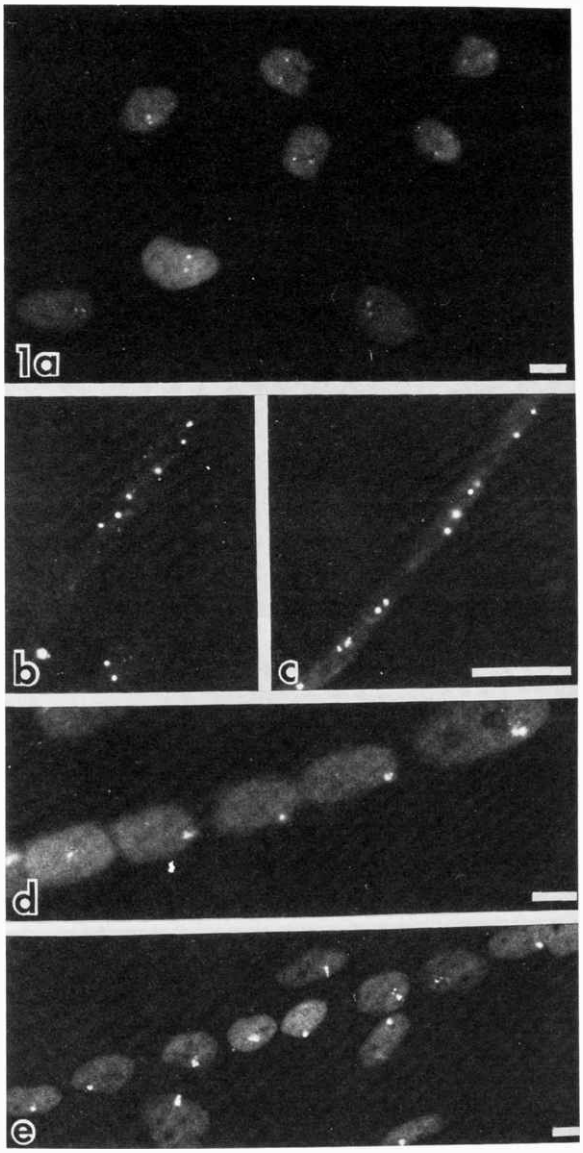


FIGURE 1 Centromeres of homologous chromosomes show no apparent orientation in isolated myoblasts. (*a*) Human male myoblasts were hybridized in situ with α-satellite DNA probes for chromosome 12. Bar, 10 μm. In myotubes, homologous centromeres align parallel to the long axis of the myotube (*b,c*). Myotubes formed in vitro by the fusion of human myoblasts were hybridized in situ with α-satellite DNA probes for chromosome 1 (*b*) and chromosome 18 (*c*). Bars, 50 μm. The centromere of the X chromosome occupies a similar position in sister nuclei of myotubes (d,e). Human myotubes were hybridized in situ with X-chromosome-specific α-satellite DNA. Bars, 10 μm.

have used RNA polymerase II inhibitors to examine whether the nuclear organization of splicing factors was dependent on active transcription. In one such study, cells were incubated in medium containing the adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). DRB is known to decrease pre-mRNA synthesis in HeLa cells by 70% and to inhibit the appearance of mRNA in the cytoplasm by more than 95% (Sehgal et al., *Cell 9:* 473 [1976]). Upon incubation of cells with DRB at a concentration of 25 μg/ml for 2 hours, the nuclear speckles round up and the connections between speckles are lost. This is similar to the change observed with α-amanitin treatment under conditions that inhibit RNA polymerase II. However, unlike α-amanitin, the effect of DRB on the organization of splicing factors was found to be reversible within 30 minutes of removal of the drug. These data demonstrated that the organization of splicing factors in the cell nucleus is dependent on RNA polymerase II activity. When such cells were examined at the electron microscopic level, a significant decrease in the number of perichromatin fibrils was observed as compared to control cells. These data support the idea that the connections observed between the larger speckles represent small nuclear ribonucleoproteins (snRNPs) associated with perichromatin fibrils or sites of nascent transcripts. These findings are in agreement with previous studies that showed [^{3}H]uridine incorporation as well as heterogeneous nuclear (hn)RNP and snRNP antigens to be associated with perichromatin fibrils (Bachellerie et al., *Eur. J. Biochem. 58:* 327 [1975]; Fakan et al., *J. Cell Biol. 98:* 358 [1984]). Therefore, the organization of splicing factors in the nucleus is a reflection of the transcriptional activity of the cell.

Use of Antisense Probes to Examine RNA Splicing In Vivo

R. O'Keefe, D.L. Spector

To assess the effect of inhibition of pre-mRNA splicing on the organization of splicing factors, antisense DNA probes were microinjected into living cells. Oligonucleotide probes of 20 bases (5′-CTCCCCT GCCAGGTAAGTAT-3′), complementary to the region of U1 snRNA that base pairs with the 5′ splice site of pre-mRNA and that encompasses sequences previously shown to inhibit pre-mRNA splicing in vitro, were used. When this oligonucleotide probe was microinjected into the cytoplasm of

HeLa cells, the oligonucleotide entered the nucleus, and within 1 hour, changes were observed in the organization of the speckled pattern. Similar to what was observed using drugs that inhibit RNA polymerase II, antisense oligonucleotides targeted to U1 snRNA caused the speckles to round up, and the connections between speckles were no longer visible (Fig. 2A). However, when a control oligonucleotide that has no complementarity to splice sites or regions of interaction between snRNAs (5′-TCCGGTACC ACGACG-3′) was microinjected into cells, no change in the organization of splicing factors was observed (Fig. 2C). Therefore, interfering with the interaction of U1 snRNP with the 5′ splice site of pre-mRNAs resulted in a reorganization of splicing factors. Experiments are under way to determine if in vivo pre-mRNA splicing was inhibited in these experiments. Microinjected DNA oligonucleotides that hybridize to RNA sequences have been shown to stimulate RNase H activity, which results in degradation of the RNA-DNA hybrid (Akhtar and Juliano, *Trends Cell Biol. 2:* 139 [1992]). Upon RNase H cleavage of the RNA portion of the U1-oligo complement, splicing is thought to be inhibited. Splicing factors then return to storage and/or assembly sites (interchromatin granule clusters) awaiting the initiation of new pre-mRNA synthesis or synthesis of new snRNAs and snRNPs. These data suggest that active pre-mRNA splicing occurs at the perichromatin fibrils and that factors shuttle between storage and/or assembly sites (interchromatin granule clusters) and sites of active transcription (perichromatin fibrils).

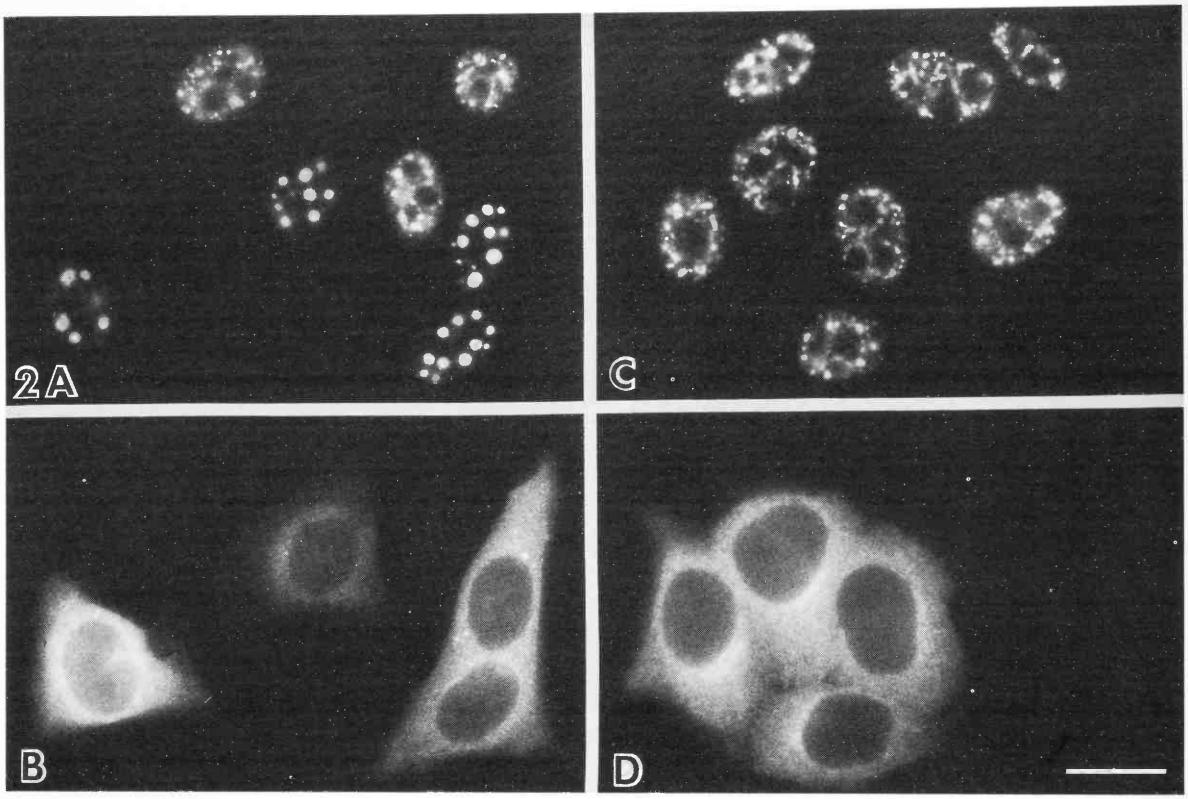
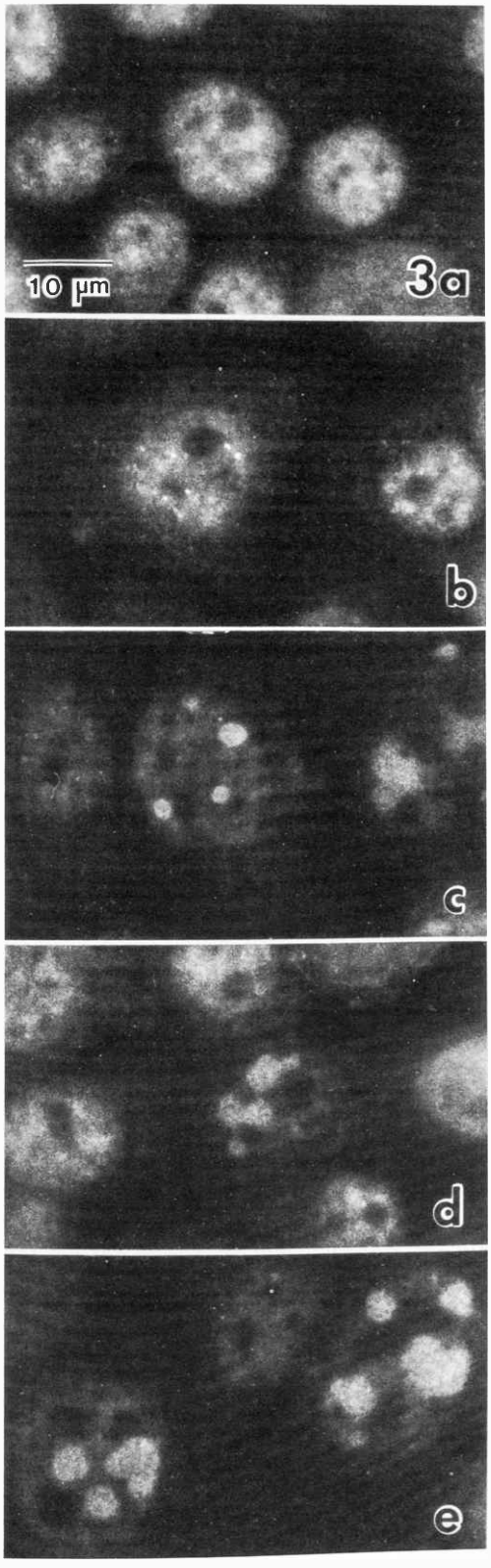


FIGURE 2 Microinjected oligonucleotides targeted to inhibit pre-mRNA splicing result in a reorganization of the splicing factor SC-35. HeLa cells were microinjected with either a specific oligonucleotide targeted to inhibit the interaction of U1 snRNP with the 5′ splice site of pre-mRNA (*A,B*) or a nonspecific oligonucleotide that should not inhibit splicing (*C,D*). Immunofluorescent labeling with an antibody to the splicing factor SC-35 2 hr after microinjection (*A,C*) revealed that the oligonucleotide targeted to disrupt the interaction of U1 snRNP with the 5′ splice site (*A*) resulted in a reorganization of SC-35, whereas microinjection of the control oligonucleotide (*C*) had no effect of the localization of SC-35. Oligonucleotides were coinjected with Texas Red conjugated dextrans (*B,D*) to mark the cytoplasm of microinjected cells. Bar, 20 μm.



How Does the Cell's Splicing Machinery Respond to the Introduction of Exogeneous DNA Templates?

L.F. Jiménez-García, D.L. Spector

We have used adenovirus 2 (Ad2) as a model system to study the spatial and temporal relationship of transcription and pre-mRNA processing in the mammalian cell nucleus. The localization of viral RNA sequences were examined throughout the infection process and compared to the distribution of several host-cell factors that are involved in transcription, pre-mRNA splicing, and packaging RNA transcripts. Ad2 RNA was first visualized in the cell nucleus as 6–12 dots at 7 hours postinfection. This time coincides with the onset of viral DNA replication and the switch from the early to the late stage of infection. These dots were shown to develop, over a 24-hour time course, into an elaborate series of rings and large dots that occupy a majority of the nuclear volume. We have previously reported that at 10–14 hours postinfection, pre-mRNA splicing factors are shuttled to the sites of Ad2 RNA where they colocalize. We extended these previous studies to determine if RNA polymerase II and proteins involved in pre-mRNA packaging are also shuttled to these sites of Ad2 RNA. In uninfected HeLa cells, RNA polymerase II is diffusely distributed throughout the nucleoplasm (Fig. 3a). However, upon Ad2 infection, RNA polymerase II is shuttled to new sites of viral RNA, indicative that these sites represent the sites of active transcription (Fig. 3b–e). We were next interested in determining whether proteins involved in packaging of nascent RNA transcripts would also reorganize upon Ad2 infection. We evaluated the distribution of the hnRNP C_1 and C_2 proteins by using an antibody that reacts specifically with these proteins (Choi and Dreyfuss, *J. Cell Biol. 99:* 1997 [1984]). In uninfected cells, the C proteins are diffusely distributed throughout the nucleoplasm, excluding the nucleolus (Fig. 4a). Similar to what was observed with SC-35, snRNPs, and RNA polymerase II, at 10–14 hours

FIGURE 3 Localization of RNA polymerase II after adenovirus infection. Upon adenovirus infection, RNA polymerase II is sequestered from its diffuse nuclear distribution (*a*) and over the time course of infection (*b–e*) localizes at sites of Ad2 RNA, suggesting that these are the sites of Ad2 transcription.

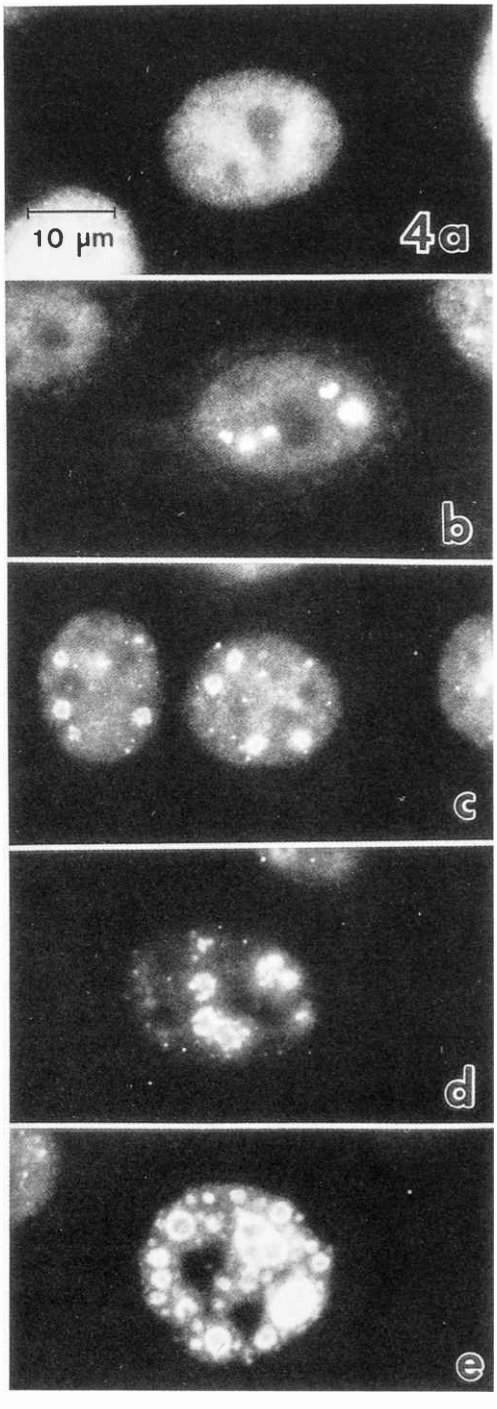


FIGURE 4 hnRNP proteins are also shuttled to sites of adenovirus transcription. In uninfected cells, hnRNP C proteins localize in a diffuse nuclear distribution (*a*). Upon adenovirus infection, the C proteins are shuttled to the sites of viral RNA (*b–e*) where they colocalize with RNA polymerase II, snRNPs, and SC-35.

into the infection process, we began to see a redistribution of C proteins to the sites of Ad2 RNA localization (Fig. 4b–e). However, the La protein which is not involved in RNA polymerase II transcription or splicing remained diffusely distributed throughout the nucleoplasm. Furthermore, in COS-1 cells transiently transfected with a plasmid containing a portion of the rat tropomyosin gene (Guo et al., *Genes Dev. 5:* 2096 [1991]), a similar shuttling of splicing factors to sites of new RNA synthesis was observed (Fig. 5).

Our studies on the localization of pre-mRNA splicing factors and RNA substrates suggest that the nucleus is highly organized into macromolecular domains associated with particular nuclear functions. At least two potential mechanisms, a *scanning mechanism* or a *recruiting mechanism*, can be proposed to account for the transcription-dependent shuttling of both transcription and pre-mRNA processing factors upon adenovirus infection. In the scanning mechanism, factors would continuously diffuse throughout the nucleus in a soluble form or move on components of a nuclear matrix. When these factors reached a potential active site, they would dock and transcription and processing would occur. Evidence has been provided that both active sites of transcription, splicing components, pre-mRNA, as well as poly(A)$^+$ RNA, are associated with the nuclear matrix. However, the continuous movement of factors in living cells has not yet been demonstrated. In the recruiting mechanism model, factors would be associated with specific storage and/or assembly sites in the nucleus. Prior to or at the initiation of transcription, these factors would be recruited to the active sites of transcription by another factor or chaperon molecule. Evidence to support this model comes from previous studies which showed that a subpopulation of splicing factors are localized to interchromatin granule clusters in mammalian cell nuclei. These clusters contain little labeled RNA after short pulses with [^{3}H]uridine, suggesting that they may not represent active sites of transcription. Therefore, in uninfected cell nuclei, splicing factors are localized to both sites of active transcription (perichromatin fibrils) and storage and/or assembly sites (interchromatin granule clusters). Furthermore, upon introduction of new transcription sites into the cell nucleus, one observes a concomitant decrease in the signal intensity of splicing factors at host-cell speckles with an increase at new active sites of viral transcription, whereas the overall level of snRNP proteins remains constant

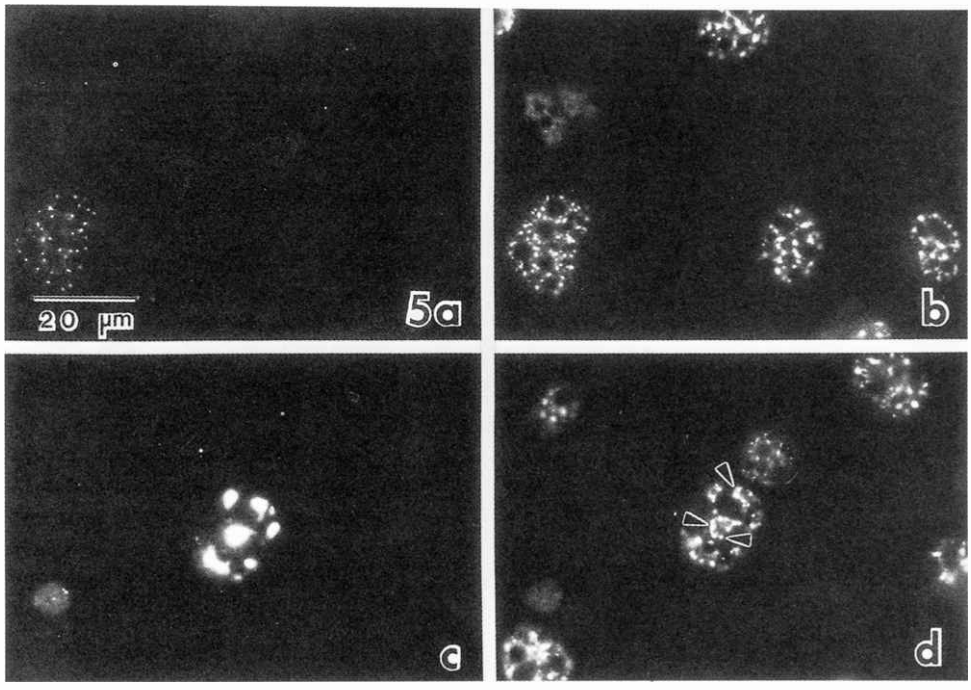


FIGURE 5 Splicing factors are shuttled to sites of β-tropomyosin transcription. Colocalization of β-tropomyosin RNA (*a,c*) and SC-35 (*b,d*) in transiently transfected COS-1 cells. At 24–48 hr posttransfection, β-tropomyosin RNA is localized in numerous fine dots within the nucleoplasm (*a*). Splicing factors appear to be localized in smaller and more numerous speckles as compared to cells that were not transfected (*b*). At later time points posttransfection, the β-tropomyosin RNA is localized in fewer nuclear regions which are larger in size (*c*), and splicing factors are associated with these regions (arrowheads in *d*).

throughout the infection process. These findings strongly suggest that there are signals generated in the nucleus that regulate the compartmentalization of factors to nuclear regions where they will be functioning. Identification of these signaling mechanisms will be key to understanding the integration of a variety of functional events that occur within the boundaries of the nuclear envelope.

mRNP Transport

S. Huang, D.L. Spector

We have been interested in how mRNAs travel from their site of active transcription to the nuclear envelope. Several previous studies showing that transcripts localize in a track that extends toward or to the nuclear envelope (Lawrence et al., *Cell 57:* 493 [1989]; Huang and Spector, *Genes Dev. 5:* 2288 [1989]) suggest that at least some RNAs may be actively transported within the cell nucleus. Unfortunately, a clean and reliable functional assay to evaluate mRNP export in mammalian cells is not available. We have recently used a temperature-sensitive mutant cell line as a means of studying RNA transport and potentially identifying factors involved in transport. TsBN2 cells are baby hamster kidney cells that contain a temperature-sensitive mutation in the RCC1 gene (Nishimoto et al., *Cell 15:* 475 [1978]). If G_1 cells are placed at the restrictive temperature, they arrest in this phase of the cell cycle. If S-phase cells are shifted to the restrictive temperature, DNA condensation occurs. However, mRNA export was also reported to be blocked in this cell system. We were first interested in determining how defects in the RCC1 gene would affect the nuclear organization of pre-mRNA splicing factors and poly(A)$^+$ RNA. Within 2–4 hours of shifting G_0/G_1 cells to the nonpermissive temperature, splicing factors and poly(A)$^+$ RNA reorganized from a

speckled nuclear distribution to 1–8 round foci within the cell nuclei. Staining with the DNA fluorochrome Dapi showed that DNA was not condensed in these cells, suggesting that the reorganization of splicing factors is not a result of DNA condensation. We are examining the possibility that RCC1 is an upstream regulator whose ultimate effect may result in maintenance of the speckled nuclear regions to fulfill the functions of transcription, pre-mRNA splicing, and mRNP export. In addition, we are evaluating the level of pre-mRNA splicing in this cell system at the permissive and restrictive temperatures. Future studies will address the identification of factors that may be involved in mRNP transport.

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POSTTRANSLATIONAL MODIFICATIONS AND APOPTOSIS

S.D. Patterson N. Bizios N. Sareen
P. D'Andrea Z. Yu
J. Horwitz

To understand the function of a gene product fully, it is essential to characterize the endogenous protein to as great an extent as possible. This includes determining the posttranslational modifications of the protein under investigation and whether these modifications are a dynamic process. Our laboratory is continuing to pursue this problem both on a "global" scale through our affiliation with the QUEST Protein Database Center using two-dimensional (2D) gels and at the level of specific protein molecules using a variety of techniques including accurate mass determination by laser desorption–mass spectrometry (LD-MS). In last year's Annual Report, we described our attempts to arrest Jurkat T lymphoblasts in a cell-division cycle-dependent manner with the drug lovastatin. It was found that these cells were not arrested with lovastatin (at high concentrations), but they were induced to undergo apoptosis, or programmed cell death. We have decided to pursue this interesting biological phenomenon, specifically in an attempt to elucidate the putative common signaling pathway that leads to apoptotic induction in many cell types. Our experience in protein identification and characterization will assist us to this end.

The highlights of this year have been the demonstration of our model systems to achieve enrichment of cells in different stages of apoptosis for subsequent characterization and the characterization of proteins using LD-MS. Apoptosis is characterized by a series of physiological events that result in cells condensing their chromatin, exhibiting marked surface "blebbing" and increased buoyant density (due to shrinkage and exclusion of water), cleaving their DNA into nucleosome-sized fragments, and finally fragmentation of the cells into small, membrane-bound apoptotic bodies. In vivo, these apoptotic cells and bodies are rapidly taken up by phagocytic cells with no ensuing inflammation and are disposed of in a man-

ner that does not expose the surrounding tissue to degradative enzymes. This process is therefore a very neat way to eliminate unwanted cells. It has been shown recently that many effective anticancer drugs work through the induction of apoptosis in the target cancer cells. A wide variety of agents can induce apoptosis, but nearly all of them cause the cell to undergo the same process. Therefore, there may be a common signaling process involved (which in some cases can be short-circuited). It is our aim to attempt to elucidate components of this pathway to better understand the process of apoptotic induction.

The protein chemistry component of the laboratory has been involved with investigating the utility of LD-MS to determine accurately the mass of proteins and their peptide cleavage products, eluted from a protein-blotting membrane following electrophoresis (both 1D and 2D gels) and electrotransfer. Accurate mass analysis is a key component of posttranslational modification characterization of endogenous proteins and can also be used for identification. We have demonstrated the ability to obtain a mass spectrum from a single protein spot on a preparative 2D gel. This work is also assisting us with our studies on apoptosis as it is being used in conjunction with other protein chemistry methods to identify and characterize proteins involved in the induction of apoptosis.

This was an exciting year of change for the 2D Gel Lab Core Facility both in location and with incorporation of new leading edge detection technology, which is reported in detail below. Our only staff change this year was Zailin Yu, who started our cell culture work. He left for the Fels Institute at Temple University School of Medicine half way through the year and was replaced by Peter D'Andrea.

Induction of Apoptosis in Jurkat T Lymphoblasts

S.D. Patterson, J. Horwitz, P. D'Andrea, Z. Yu

Inhibition of the rate-limiting enzyme of the cholesterolgenesis pathway, HMG CoA reductase, in some cells has been shown to cause a reversible G_1 cell cycle phase arrest. We set out to determine whether the cell line, which we are using in the initial development of our human 2D gel protein database (see QUEST Protein Database Center, this section), could be arrested in a stage of its cell cycle. The aim of this arrest was to establish a means of synchronizing the cells for subsequent release and analysis of stages of the cell cycle. Studies of this drug demonstrated no obvious G_1 cell cycle phase arrest, but upon flow cytometric analysis of the DNA content of these cells, a number of cells displaying DNA content less than G_0/G_1 ($2n$) were observed. Further microscopic analysis of the cells, as well as separation of isolated DNA from these cells, confirmed the suspicion that a portion of the cells were undergoing apoptosis in response to high concentrations of the drug. Through the use of Percoll gradients, apoptotic cells can be separated from nonapoptotic cells because of their increased buoyant density. We have conducted labelings of the separated cells (whole-cell lysates, nuclei-enriched fractions, and their postnuclear supernatants) following [^{35}S]methionine and more recently [^{32}P]orthophosphate labelings.

A somewhat unexpected result of this study is that the 2D-gel-labeling pattern of the apoptotic cells is almost indistinguishable from that of untreated cells, despite their markedly altered morphology. The few protein differences are being examined further, together with other biochemical techniques such as kinase renaturation of 1D-gel-separated proteins and immunoblotting with antisera to known intracellular signaling molecules. From our studies so far, one of the few proteins that displays decreased synthesis is an acidic protein that has a relative mass of 40 kD. This protein was identified as numatrin (B23) both by using monospecific antiserum (from Dr. Olson, University of Mississippi Medical Center) and by obtaining the amino acid sequence of the protein.

The sequence data were obtained by separation of a number of whole-cell lysates by preparative 2D gels, followed by blotting to nitrocellulose. The stained, excised nitrocellulose spots were digested with trypsin. One of the collected peptides was sequenced by Dr. R. Kobayashi (see Tumor Viruses Section) and yielded the sequence MTDQEAIQDL, confirming the identity of the protein as numatrin (B23). This protein, which undergoes increased synthesis upon mitogenic stimulation of lymphocytes and displays markedly increased concentration in leukemic cells, is thought to be involved in assembly of preribosomal particles, intranuclear transport, and translocation of ribosomal components across the nuclear envelope. Whether the decreased synthesis of numatrin (B23) is a result of apoptotic induction or is an important early feature has not yet been determined. We are currently using additional agents to induce apoptosis in these cells.

"Activation-induced" Apoptosis in Human T Lymphoblasts

S.D. Patterson [in collaboration with P. Krammer and M. Peter, Deutsches Krebsforschungszentrum (DKFZ), Germany]

Apoptosis, or programmed cell death, can be induced very rapidly (~30 min) in some human cells that possess the APO-1 surface antigen (the murine homolog being the Fas antigen). This antigen is recognized by the anti-APO-1 antibody raised and characterized in Professor P. Krammer's laboratory at DKFZ. We are currently collaborating with Professor Krammer and Dr. Peter to search for alterations in the pattern of [^{35}S]methionine or [γ-^{32}P]ATP labeling. Dr. Peter visited our laboratory for a week in August to provide the first samples for this study and to discuss the aims.

Cells prelabeled with [^{35}S]methionine and those labeled during the activation are being analyzed to search for both posttranslational modifications that result in altered 2D electrophoretic mobility and new protein synthesis, respectively. Alterations in phosphate labeling are being searched for using [γ-^{32}P]-ATP labeling of cells permeabilized following anti-APO-1 activation. This study has already revealed the presence of only a few alterations in phosphate labeling. We are currently examining the ^{35}S/^{32}P mixes to determine whether the newly phosphorylated proteins can be found in the [^{35}S]methionine-labeled patterns or whether the proteins are at too low a level to be detected in the unfractionated post-nuclear supernatants. The aim of the project is to then identify the proteins that display altered 2D electrophoretic mobility or altered phosphorylation upon anti-APO-1 activation-induced apoptosis and to determine whether any of these components are the same as those found in our own study on apoptotic induction.

LD-MS of 2D-gel-separated Proteins for Identification and Characterization

S.D. Patterson [in collaboration with B.T. Chait and W. Zhang, The Rockefeller University, New York]

The initial aim of this research is to determine whether accurate mass analysis of both intact and fragmented proteins is possible following blotting of proteins separated by either 1D or 2D gel electrophoresis. Using both a commercial LD-MS instrument, the Kompact MALDI III (courtesy of Kratos Analytical, Ramsey, New Jersey), and one of the original LD-MS instruments (Professor Brian Chait, Rockefeller University), we have been examining whether this approach is feasible. In last year's Annual Report, we outlined the characterization of a new protein-blotting membrane, Immobilon-CD. This membrane has the advantage that recovery of proteins from it is both efficient and in a form compatible with further sensitive analyses. We have already established that there are components being released from the Immobilon-CD membrane that cause suppression of the LD-MS signal, unless the membrane is extensively washed with basic organic solvent, suggesting that the contaminating component may be residual SDS. Using this technique, we have been able to produce a spectrum from myoglobin separated by a 1D gel and blotted to Immobilon-CD. Following a rapid (2-min) cyanogen bromide (CNBr) cleavage (a reagent that cleaves after methionine residues), a spectrum that displays all of the expected fragments including the partially digested fragments was observed. The mass of these fragments is very close to that expected, but there is still more work to be done to achieve very high mass accuracy.

To test the system further, we subjected the nucleolar protein, numatrin (B23) (which was separated by preparative 2D gel electrophoresis), to the same rapid CNBr cleavage. The designation was made on the basis of its identification by a monospecific antiserum and amino acid sequence analysis (see above Induction of Apoptosis in Jurkat T Lymphoblasts). Numatrin (B23) is in relatively high abundance in leukemic cells and displays an acidic isoelectric point and an aberrant relative molecular mass of 40 kD (the calculated mass is 32 kD). CNBr produced a mass spectrum that was consistent with the protein being numatrin (B23), thereby confirming the assignment and also demonstrating that this technology can be used on proteins separated by 2D gel electrophoresis. Similar results were obtained using the commercial instrument, the Kompact MALDI III, at Kratos Analytical, in Ramsey New Jersey; the smoothed spectrum obtained from a single 2D gel protein spot of numatrin (B23) is shown in Figure 1.

Development of this technology will allow a rapid means for identification of proteins separated by 2D gels, at levels less than that required for sequence analysis, and most importantly provide data for sub-

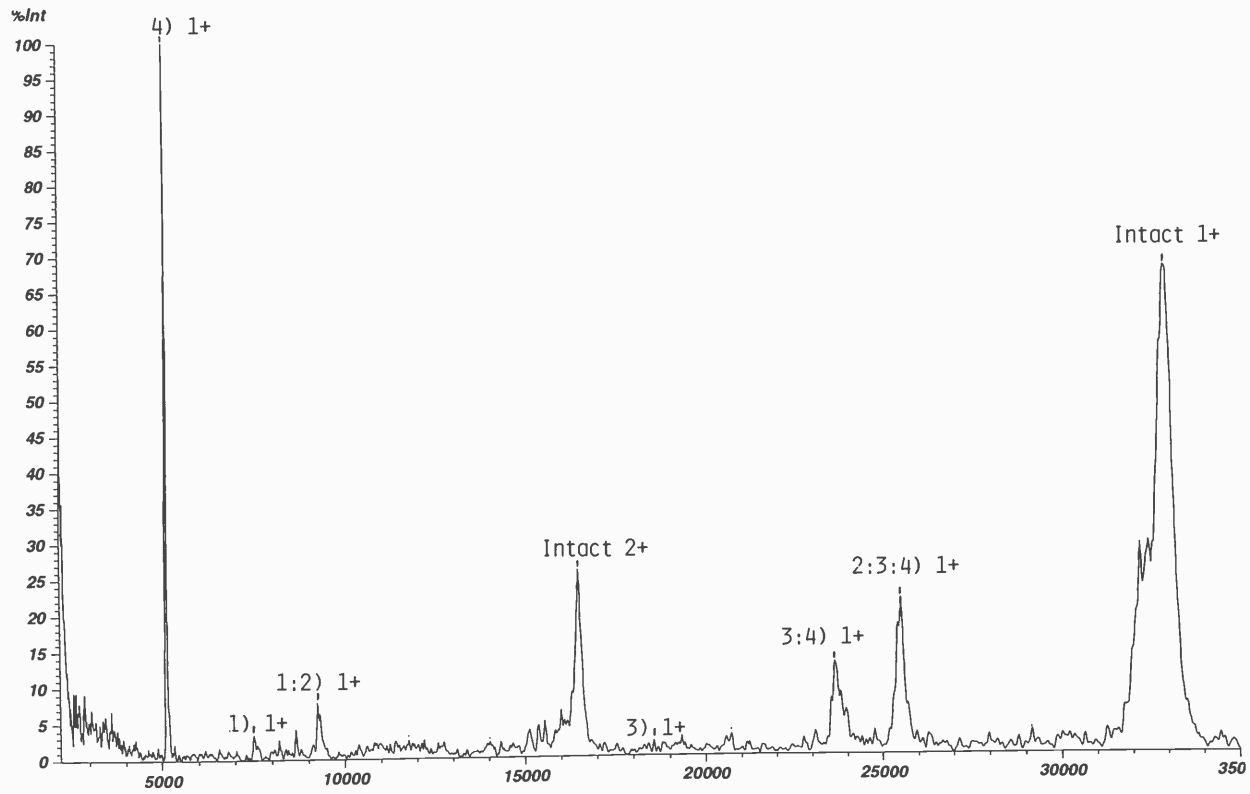

FIGURE 1 A smoothed LD-MS spectrum of a rapid (5-min) CNBr digest of a single protein spot from a preparative 2D gel obtained using the Kompact MALDI III. Approximately 4% of the material eluted from the excised, cut up, and CNBr-treated protein spot was applied to the probe. The mass spectrum shows the relative mass region of 2,000–35,000 daltons; no peaks of higher mass were observed. The peaks are labeled as intact or with their fragment numbers (from partial cleavages) and charge state (1+ = $[M + H]^{1+}$, 2+ = $[M + 2H]^{2+}$). Numatrin (B23) has methionine residues at positions 1, 5, 7, 9, 65, 81, 251, and 278. The fragment numbers corresponding to the cleavage products are as follows: 1 = 1 + 2-5 + 6-7 + 8-9 + 10-65 (the partial cleavage yielding fragment 1-65), 2 = 66-81 (complete cleavage fragment), 3 = 82-251 (complete cleavage fragment), 4 = 252-278 + 279-294 (the partial cleavage yielding fragment 252-294).

sequent posttranslational modification analysis. This will also allow us to peptide map more accurately proteins that have been located in our 2D patterns following in vitro transcription/translation. For example, within our Jurkat pattern, we have identified the vertebrate actin-related protein, actin-RPV (Lees-Miller et al., *Nature 359:* 244 [1992]), in collaboration with Drs. Lees-Miller and Helfman (see Molecular Genetics of Eukaryotic Cells Section) using this approach, but with gel eletrophoretic analysis of the peptide fragments, not LD-MS. In this case, following the translation reaction, the labeled protein products were separated on a 2D gel alone and mixed with a whole-cell lysate (or appropriate enriched fraction) (Fig. 2). After location of a comigrating protein, both the in vitro translated protein and the endogenous protein were excised from the gel and subjected to limited peptide mapping and gel separation to determine whether the correct protein had been located. As the cDNA had been sequenced, the protein sequence can be deduced and used to assist in confirming the peptide map obtained (Fig. 2). However, the masses obtained by gel electrophoresis are only approximate, whereas using LD-MS for accurate peptide mapping, and correlating this information with sequence information, provides valuable information for scientists interested in characterization of endogenous proteins.

2D Gel Laboratory Core Facility

S.D. Patterson, N. Bizios, N. Sareen

There has been a significant change to the operation of the Cold Spring Harbor Laboratory 2D Gel Lab

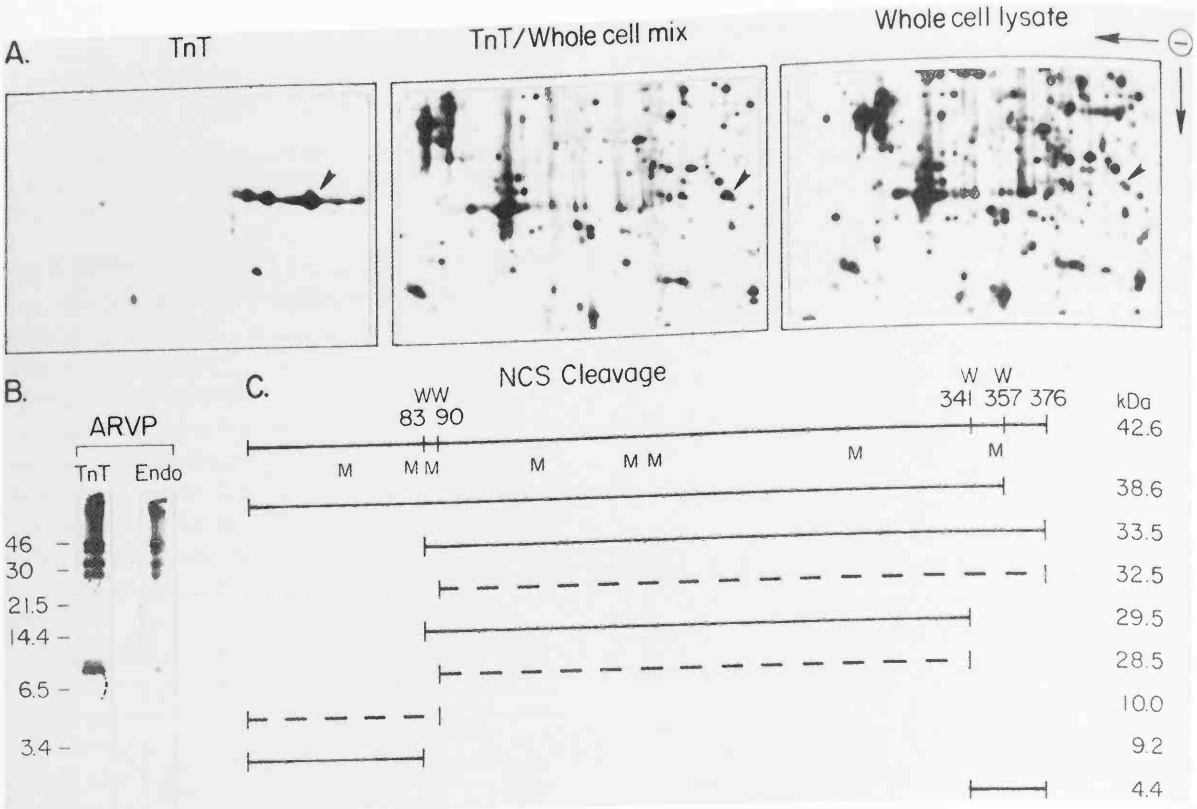

FIGURE 2 Identification of the actin-related vertebrate protein, actin-RPV, gene product in Jurkat cells. Portions of the 2D gels (pI range of ~5.0–6.5, and relative molecular mass of 60–40 kD) are shown in *A*. (*Left panel*) In-vitro-translated actin-RPV (TnT); (*middle panel*) mixture of the TnT and Jurkat whole-cell lysate (TnT/Whole cell mix); (*right panel*) Jurkat whole-cell lysate (Whole cell lysate). Arrowheads in all panels indicate the most basic in-vitro-translated actin-RPV gene product (and the endogenous protein). The identity of the TnT gene product and the endogenous cellular protein, following excision from the gel, was then confirmed by 1D NCS (*N*-chlorosuccinimide, a reagent that cleaves after tryptophan residues) peptide mapping, shown in *B* together with the expected cleavage products of the actin-RPV protein shown schematically in *C*. In *C*, M represents the positions of the eight methionine residues, W represents the four tryptophan residues, and the dashed lines are those peptides that were barely visible in *B*.

Core Facility this year with the move from our original location in the McClintock building to Demerec in early June. Fortunately, there was only minimal downtime associated with this move and our thanks go to Buildings and Grounds for helping us in this regard. The move to a more compact laboratory, change of our method of detection to storage phosphor technology, and some operational changes have allowed improvement of the existing service through more rapid turnaround of the results. Dependent on the number of samples that are waiting to be run, many gel images are available within about 1 week of submission. This increase in turnaround is primarily due to the use of the leading edge imaging technology, storage phosphor imaging, and the assistance of the QUEST staff in providing software support for efficient archiving of the image data. These images are in digitized form, which is advantageous for subsequent quantitative analyses.

We ran just over 2100 gels this year; almost 98% of these were for Cold Spring Harbor Laboratory staff (and their collaborators), and the remainder were for external users. In addition, gels were also run for quality control and testing. This core facility continues to provide an important service to the Laboratory scientists, as well as to the general scientific community.

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QUANTITATIVE REGULATORY BIOLOGY

B.R. Franza G. Mak, B.-C. Ma, M. Neumann, A. Calzolari, J. Scheppler, Y. Li, H.-R. Tan, G. Niu

During the time I have been at Cold Spring Harbor Laboratory, my colleagues and I have demonstrated our ability to identify and study cellular regulatory proteins. We have made use of two-dimensional gel technology in this effort and have demonstrated the extent to which the computerization of the two-dimensional gel data is necessary—there are many regulatory proteins and both the processes that they control and their own regulation are dynamic. For example, the rates of synthesis, posttranslational modification, and interaction with other cellular proteins comprise a few of the modalities of regulation of a particular protein. As cells change their growth and/or differentiation status, the different protein states change. We are beginning to learn how to keep track of sufficient amounts of dynamic information about these molecular events to describe the mechanisms that regulate the expression and function of regulatory proteins. These studies are expected to reveal the nature of intermolecular relationships that determine the growth and differentiation status of the organism being investigated.

We have studied different classes of cellular regulatory proteins involved in the growth control of mammalian cells. Lymphoblasts and hematopoietic progenitor cells are the normal human tissues we most commonly use to study the molecular mechanisms regulating cell proliferation and differentiation. To characterize regulatory proteins, we have focused on three processes. One is the effect expression of oncogenes has on the entire complement of proteins present in a particular cell type. The second is the induction of protein alterations when quiescent cells are stimulated to proliferate. These studies are directed at determining the molecules involved in conveying signals to the genome and the earliest responses of the genome to these signals. The third process is the control of gene expression at the level of regulation of transcription of mRNA encoding genes. The Quest II protein database system is the tool on which we rely heavily for performing such a comprehensive qualitative and quantitative analysis of regulatory proteins. The goal, implicit in all of our studies, is to construct a molecular description of the networks involved in regulating transcription and other genome responses to a change in the growth state of the cell. The individuals listed above work as a group, with those contributing most to a particular project being recognized by first authorship of published reports (see Publication list).

In the past year, we have extended the characterization of proteins identified to interact with a transcription control element referred to as the κB site. Several years ago, we identified a number of cellular

proteins that were recovered from cells by an affinity reaction with short, synthetic oligonucleotides containing the 10-base-pair κB motif. We have since demonstrated that one of those proteins, HIVEN86A, is the product of the human c-*rel* gene. We now know that in addition to Rel, two of the protein clusters we identified in our original studies are the products of related genes because they encode regions similar to a region in Rel. The products of these genes have been studied extensively by numerous groups and are referred to as the NF-κB transcription factor. We have recently shown that the precursor of the p50 subunit of NF-κB, called p105, associates with Rel and is posttranslationally modified by phosphorylation.

Rel is also a phosphoprotein. Subsequently, we demonstrated that Rel and p105 are phosphorylated on serine, threonine, and tyrosine. The tyrosine phosphorylation and synthesis of Rel are sensitive to induced changes in the growth state of cells, including lymphoblasts and normal neutrophils. In collaboration with Bryan Druker and colleagues at the Dana Farber Cancer Institute, we have shown that Rel is one of the most immediately tyrosine-phosphorylated substrates in a signal transduction pathway sensitive to the growth factor G-CSF (granulocyte-colony stimulating factor). G-CSF is known to result in prolonged survival and enhanced tumor cell killing by neutrophils. We have shown not only that Rel is one of the most immediately modified substrates for G-CSF, but that the event is specific for G-CSF in that other neutrophil stimulatory growth factors do not induce tyrosine phosphorylation of Rel.

We have extended the characterization of other proteins that interact with the κB site in comparative analysis of the p50 subunit of NF-κB and Rel. We have now demonstrated that certain forms of p50, within the cytoplasm of cells, bind the site in in vitro affinity precipitation assays (DNAP), that alteration in nuclear binding activity of p50 is different in different cell types independent of the signal-inducing event being studied, and that selective forms of Rel are present in the cytoplasm versus nucleus of cells, although the forms that associate with the site are present in both compartments of the cell. The dynamic nature of response-sensitive changes in synthesis, modification, and specific DNA interaction remains incompletely characterized at this point, but is sufficiently complex to suggest that experiments that do not replicate the coordinate concentration fluctuations and differential compartmentalization of p50 and Rel that we are measuring as responses to signals, whether in in vitro biochemical assays or in "whole cell" assays, are potentially incomplete.

Consistent with our interest in the dynamic response of regulatory proteins to the cells' transit through different growth states, we have begun a more extensive characterization of the kinases and phosphatases—enzymes that add or remove phosphate from specific amino acid components of proteins. One such study has been conducted in collaboration with Nick Tonks (see this section). We have been able to demonstrate that a specific type of phosphatase, protein tyrosine phosphatase 1B (PTP1B), is itself phosphorylated. The phosphorylation occurs as cells transit the cell division cycle. Cells also respond to signals induced by an activator of protein kinase C. In fact, protein kinase C modifies PTP1B in biochemical assays at the same residue identified as modified in cells treated with a major activator of protein kinase C. The task of PTP1B is to remove phosphate from tyrosine residues in other proteins, which is why it is referred to as a tyrosine phosphatase. Interestingly, through our collaboration with Nick Tonks and a graduate student in his laboratory, K. Lamontagne, we have demonstrated that PTP1B is modified by tyrosine phosphorylation. Using cell lines provided by Bryan Druker, we have also been able to demonstrate that the expression of an "activated," oncogenic form of the Abl kinase results in an apparent increase in tyrosine phosphorylation of PTP1B and an increase in the amount of PTP1B itself. This is demonstrable because the parental cells not expressing the oncogenic Abl kinase are used for controlled comparisons of protein levels and extent of phosphorylation. This is important because the mutant, oncogenic form of the Abl kinase is the result of a translocation and recombination of a portion of the coding region for another regulatory protein, Bcr, with a portion of the coding region for Abl. This Bcr-Abl fusion product is found in virtually all cases of chronic myelogenous leukemia and represents the most thoroughly studied consequence of the chromosomal abnormality referred to as the Philadelphia chromosome. The biological significance of the altered regulation of PTP1B by increased steady-state tyrosine phosphorylation is under investigation.

These highlights of our studies during the past year indicate, once again, the necessity to resolve the different forms of a particular gene product in order to discern and quantify the state changes of the protein coincident with the state changes of cells responding

to different signals and/or traversing the cell division cycle. The Quest II database of these regulatory proteins will enable us to monitor and analyze an otherwise intractably complex system. Such complex system analysis is fundamental to understanding the material basis of living organisms.

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MOLECULAR CELL BIOLOGY

D.M. Helfman C. Casciato, W. Guo, J. Kazzaz, J.P. Lees-Miller, G. Mulligan, M. Pittenger, S. Stamm, T. Tsukahara, J. Wang, S. Wormsley

During the last few years, our laboratory has focused on investigating the structure, expression, and function of the genes encoding the protein components of the cytoskeleton. These studies have led us to examine two fundamental problems in molecular and cell biology: (1) the mechanisms responsible for tissue-specific and developmentally regulated patterns of gene expression and (2) the functional significance of cell-type-specific protein isoform expression. Much of our efforts have been to understand the regulation and function of tropomyosin gene expression in muscle and nonmuscle cells. Tropomyosins are major actin-filament-binding proteins in muscle (skeletal, smooth, and cardiac) and nonmuscle cells. Actin filaments are involved in a number of fundamental cellular processes including muscle contraction, cell movement, cell division, and the generation of cell shape. Of interest to our research is the observation that different forms of tropomyosin are expressed in different cell types and tissues. Distinct isoforms are present in striated muscle (skeletal and cardiac), smooth muscle, and nonmuscle cells. We now know that at least 12 different tropomyosin isoforms are expressed from three separate genes in rat. The α-

tropomyosin (α-TM) gene encodes nine isoforms, the β-tropomyosin (β-TM) gene encodes two isoforms, and the TM-4 gene encodes a single isoform. We have been studying the α-TM and β-TM genes with particular attention to understanding the mechanisms responsible for tissue-specific alternative RNA splicing. In addition, we have begun to study alternative splicing of the clathrin light chain B gene, with an emphasis on understanding the molecular basis for neuron-specific splice site selection. We are also interested in determining the function of the various isoforms of tropomyosin in normal and transformed fibroblasts, as well as a group of isoforms that are only expressed in brain. Recently, we identified a gene in vertebrate cells that encodes a distantly related member of the actin family and have begun to study the function of this actin-like protein. Below is a summary of our studies.

cis-Acting Elements Involved in Alternative Splicing of the Rat β-Tropomyosin Gene

W. Guo, D. Helfman

Alternative RNA splicing is a fundamental process in eukaryotes that contributes to tissue-specific and developmentally regulated patterns of gene expression. We have been using the rat β-TM gene as a model system to study the mechanism of alternative splicing. The rat β-TM gene spans 10 kb with 11 exons and encodes two distinct isoforms, namely, skeletal muscle β-TM and fibroblast TM-1. Exons 1–5, 8, and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as in smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle cells. Our previous studies localized critical elements for regulated alternative splicing to sequences within exon 7 and the adjacent upstream intron. We also demonstrated that these sequences function, in part, to regulate splice site selection in vivo by interacting with cellular factors that block the use of the skeletal muscle exon in nonmuscle cells (Guo et al., *Genes Dev. 5:* 2096 [1991]). This year, we have further characterized the *cis*-acting elements involved in alternative splice site selection. We have made a chimeric minigene composed of the adenovirus major late transcription unit driven by the cytomegalovirus (CMV) promoter/enhancer, in which the β-TM exon 7 and its immediate flanking intron regions were inserted between exons 1 and 3 of the adenovirus early region. We also cloned into the heterologous minigene three mutants in the intron upstream of exon 7, termed int-3, int-5, and d3-5, that were characterized previously and found to result in the use of exon 7 in nonmuscle cells. Expression of the wild-type heterologous minigene in nonmuscle cells results in spliced products containing adenovirus exon 1 spliced to exon 3, and exon 7 of the TM gene is not used. In contrast, mutants int-3 and int-5 led to partial use of exon 7, as shown by the splice product containing adenovirus exon 1 + β-TM exon 7 + adenovirus exon 3. Mutant d3-5 almost completely activated exon 7 in the heterologous gene. These results demonstrate that exon 7 and its immediate flanking intron sequences are sufficient to regulate the suppression of exon 7 in nonmuscle cells. To further study the mechanism responsible for the suppression of exon 7 splicing, we introduced the wild-type sequences, as well as three intron mutants into plasmid P2(7/8) that have exons 7 and 8 ligated together, and performed both in vitro and in vivo assays. Our results indicated that the blockage of exon 7 in nonmuscle cells is primarily at its 3′ splice site. We then used biotin-labeled RNA and gel-filtration chromatography to study pre-splicing complex assembly of exon 7. We studied the small nuclear ribonucleoprotein (snRNP) composition and protein components that assemble on wild-type and mutant constructs during the early stage of spliceosome assembly. We have found the binding of U1 and U2 snRNPs are similar for wild-type and mutant pre-mRNAs. These results suggest that the factors which block the 3′ splice site of exon 7 do not function by interfering with the binding of U1 and U2 snRNP particles to the pre-mRNA.

HeLa Cell Nuclear Factors Block the Skeletal Muscle-type Splicing of β-Tropomyosin Pre-mRNA

G. Mulligan, D. Helfman

The β-TM transcript contains a mutually exclusive internal cassette, in which exon 7 is spliced into mRNA in skeletal muscle, and exon 6 is used in all other tissues. Our studies demonstrate that in nonmuscle cells, exons 6 and 7 do not compete for splice site recognition, but instead cellular factors block the use of exon 7. Exon 7 sequences are critical to block-

age, as their mutation can activate splicing in nonmuscle cells. Within the upstream intron, a long polypyrimidine tract positions the remote branchpoint associated with splicing of exons 5 to 7, and the intron regulatory element (IRE) located between exon 7 and the upstream polypyrimidine tract is also necessary for nonmuscle blockage of exon 7. Using a mobility shift assay, we purified an IRE-specific binding activity and found that it is identical to the polypyrimidine-tract-binding protein (PTB). Furthermore, we have shown a direct correlation between reduced PTB binding and activation of exon 7 (Mulligan et al., *J. Biol. Chem. 267:* 25480 [1992]).

Functional analysis of cellular factors involved in exon 7 blockage has been hampered by the lack of an extract that uses the skeletal muscle exon to significant levels. We have now developed a modification in nucleus isolation that allows both HeLa cell and 293 cell nuclear extracts to utilize the 3′ splice site of exon 7. This modification alters the distribution of cellular factors between the nuclear and cytoplasmic fractions. The stimulation of exon 5 to 7 splicing may be due to the overall increase in splicing efficiency and/or the reduction of specific blocking factors. These extracts contain significant levels of PTB, and addition of more PTB did not reduce exon 7 splicing. Both affinity chromatography and immunodepletion with PTB antibodies are being used in attempts to reduce PTB levels such that possible effects on exon 7 splicing can be observed. We are also using this extract as the basis for a complementation assay to detect factors blocking exon 7 splicing. Addition of HeLa cell nuclear extract that inefficiently splices exon 7 indicates the presence of general and specific inhibitory activities. However, a partially purified fraction specifically reduces exon 7 use without inhibiting splicing of controls such as β-globin. We are purifying this activity in an effort to understand both the mechanism and specificity of exon 7 blockage.

Regulation of Alternative Splice Site Selection in β-Tropomyosin Pre-mRNA

T. Tsukahara, D. Helfman

We have been studying why the splicing of exon 5 to exon 6 in the β-TM gene requires that exon 6 first be joined to the downstream common exon (Helfman et al., *Genes Dev. 2:* 1627 [1988]). Pre-mRNAs containing exon 5, intron 6, and exon 6 are not normally spliced in vitro. We have carried out a mutational analysis to determine which sequences in the pre-mRNA contribute to the inability of this precursor to be spliced in vitro. Interestingly, we find that mutations in two regions of the pre-mRNA lead to activation of the 3′ splice site of exon 6, without first joining exon 6 to exon 8. First, introduction of a nine-nucleotide poly(U) tract upstream of the 3′ splice site of exon 6 was sufficient to permit the splicing of exon 5 to exon 6 with as little as 35 nucleotides of exon 6. Second, introduction of a consensus 5′ splice site in exon 6 leads to splicing of exon 5 to exon 6. The results obtained with the latter mutation are in agreement with the exon definition model proposed by Berget (*Mol. Cell. Biol. 10:* 84 [1990]). Collectively, our studies demonstrate that three distinct elements can act independently to activate the use of the 3′ splice site of exon 6: (1) the sequences contained within exon 8 (exon-recognition sequences), (2) a poly(U) tract in intron 5, and (3) the 5′ splice site of exon 6. Using biochemical assays, we have determined that some of these sequence elements interact with distinct cellular factors for 3′ splice site utilization. Furthermore, we have also studied how these elements contribute to alternative splice site selection using precursors containing the mutually exclusive, alternatively spliced cassette composed of exons 5–8. Introduction of the poly(U) tract upstream of exon 6 and changing the 5′ splice site of exon 6 to a consensus sequence, either alone or in combination, facilitated the use of exon 6 in vitro, such that exon 6 is spliced more efficiently to exon 8. These data demonstrate that intron sequences upstream of an exon can contribute to the use of the downstream 5′ splice site. These results also demonstrate that in addition to sequences in and around exon 7 (skeletal muscle-type splice), multiple *cis*-elements surrounding exon 6 (fibroblast-type splice) contribute to tissue-specific alternative splice site selection.

Molecular Basis for the Ordered Pathway of Intron Removal in β-Tropomyosin Pre-mRNA: Identification of a Novel Activity Required for Certain 3′ Splice Sites

C. Casciato, D. Helfman

We have been studying the internal region of the rat β-TM gene that contains mutually exclusive exons 6

(fibroblast- and smooth muscle-type splice) and 7 (skeletal muscle-type splice). As described in the previous section, our studies demonstrate that three distinct elements can act independently to activate the use of the 3′ splice site of exon 6: (1) the sequences contained within exon 8 (exon-recognition sequences), (2) a poly(U) tract in intron 5, and (3) the 5′ splice site of exon 6. Using biochemical assays, we have determined that these sequence elements interact with distinct cellular factors for 3′ splice site utilization. Although HeLa cell nuclear extracts are able to splice all three types of pre-mRNAs, an S100 fraction plus SR proteins was unable to splice exon 5 to exon 6 using precursors in which exon 6 was joined to exon 8. Using an in vitro complementation assay, we have identified a novel factor that is required for the splicing of exon 5 to exon 6 with this latter precursor. We have purified this factor extensively over three chromatographic columns. In addition, we have identified a putative target site (exon-recognition element) for the interaction of this activity, herein referred to as an exon-recognition factor (ERF). The properties of the ERF and its target RNA sequences are under study.

Alternative Splicing of β-Tropomyosin Pre-mRNA in a Myogenic Cell System

Y.-C. Wang, D. Helfman

Previous studies in our laboratory on alternative splicing of β-TM pre-mRNA have indicated that use of the skeletal muscle-specific exon (exon 7) in nonmuscle cells (HeLa cells) is subject to negative regulation, due to the presence of an inhibitory activity or factor in nonmuscle cells (Guo et al., *Genes Dev. 5:* 2096 [1991]). It is possible that skeletal muscle cells simply lack this negative activity, so that they are capable of incorporating exon 7 in β-TM mRNA during pre-mRNA splicing. However, it is also possible that skeletal muscle cells express a muscle-specific factor that overcomes the negative activity, resulting in the use of exon 7. To understand the molecular basis for muscle-specific splicing of β-TM pre-mRNA, we are developing an in vitro splicing system from myogenic cells, which will then be used to study the cellular factors involved in the regulation of muscle-specific alternative splicing. The system we are using is derived from BC3H1 cells, a mouse nonfusing muscle cell line that differentiates in culture and changes from a nonmuscle to a muscle phenotype. The transition of tropomyosin isoforms expressed from the β-TM gene at the protein and mRNA levels in BC3H1 cells has been previously characterized during the differentiation process. The nonfusing muscle feature of BC3H1 cells makes the preparation of nuclear extracts from these cells easier than that from other skeletal muscle cells. We have so far successfully made nuclear extracts from undifferentiated (myoblast-stage) BC3H1 cells that are able to carry out nonmuscle-type splicing of RNA substrates transcribed in vitro from β-TM gene fragments. The conditions we have tested for the splicing reactions show that BC3H1 nuclear extracts require salt and magnesium concentrations different from those used in the nonmuscle HeLa cell system, which is conventionally used in the research field of RNA splicing. At present, we are working on the conditions of nuclear extract preparation from differentiated (myocyte-stage) BC3H1 cells for a system that supports muscle-type RNA splicing.

In addition to the in vitro splicing system, we also use other approaches including UV-cross-linking and gel-shift assays to investigate nuclear factors that might show differential binding to β-TM pre-mRNA in myoblast versus myocyte nuclear extracts and thus might mediate muscle-specific splicing. Furthermore, through the analyses by Western blot assay performed on BC3H1 myoblast and myocyte lysates, we are studying the protein levels of a variety of factors that are known to have effects on alternative splicing in other gene systems.

Regulation of Neuron-specific Alternative RNA Splicing

S. Stamm, D. Helfman [in collaboration with Diana Casper, Mount Sinai School of Medicine]

We have determined the gene structure of clathrin light chain B and the expression pattern of its neuron-specific exon, termed exon EN (Stamm et al. 1992). To study the molecular basis for neuron-specific splice site selection, we are analyzing the regulatory sequences using minigene constructs that are transfected into primary neuronal cultures from rat mesencephalon and HeLa cells. We constructed a minigene of exon EN and its flanking exons EIV and EV. This

minigene faithfully reflects the splicing pattern of the endogenous gene. Deletion analysis of intron sequences upstream and downstream from the neuron-specific exon revealed that the sequences required for neuron-specific splice site selection are located in the alternatively spliced exon and its flanking intron sequences. We have identified an eight-base-pair motif that is conserved between the neuron-specific exon EN in clathrin light chain B and exon 9c in the α-TM gene (see also Fig. 1). Minigenes containing mutations in this sequence in the clathrin minigene no longer use the neuron-specific exon in neuronal cultures, indicating that this sequence is involved in alternative splice site selection. The neuron-specific exon EN is normally excluded in all cell types other than neurons. When we were analyzing the sequence of the splice sites of exon EN, we noticed that both the 5′ and 3′ splice sites deviate from the mammalian consensus sequence. To address the question of why EN is excluded, we mutated its 5′ and 3′ splice sites to match the consensus 5′ and 3′ splice site sequences. All constructs that have at least one consensus splice site use exon EN in HeLa cells. The 5′ splice site seems to be more important for its use than the 3′ splice site. When both splice sites are in consensus, the exon is changed into a constitutively spliced exon. Our data suggest that the neuron-specific exon EN is not used in most cells because its splice sites are not recognized. On the other hand, neurons would be expected to express a factor that binds to the alternatively spliced exon and thereby promote its use. Using UV cross-linking, we have

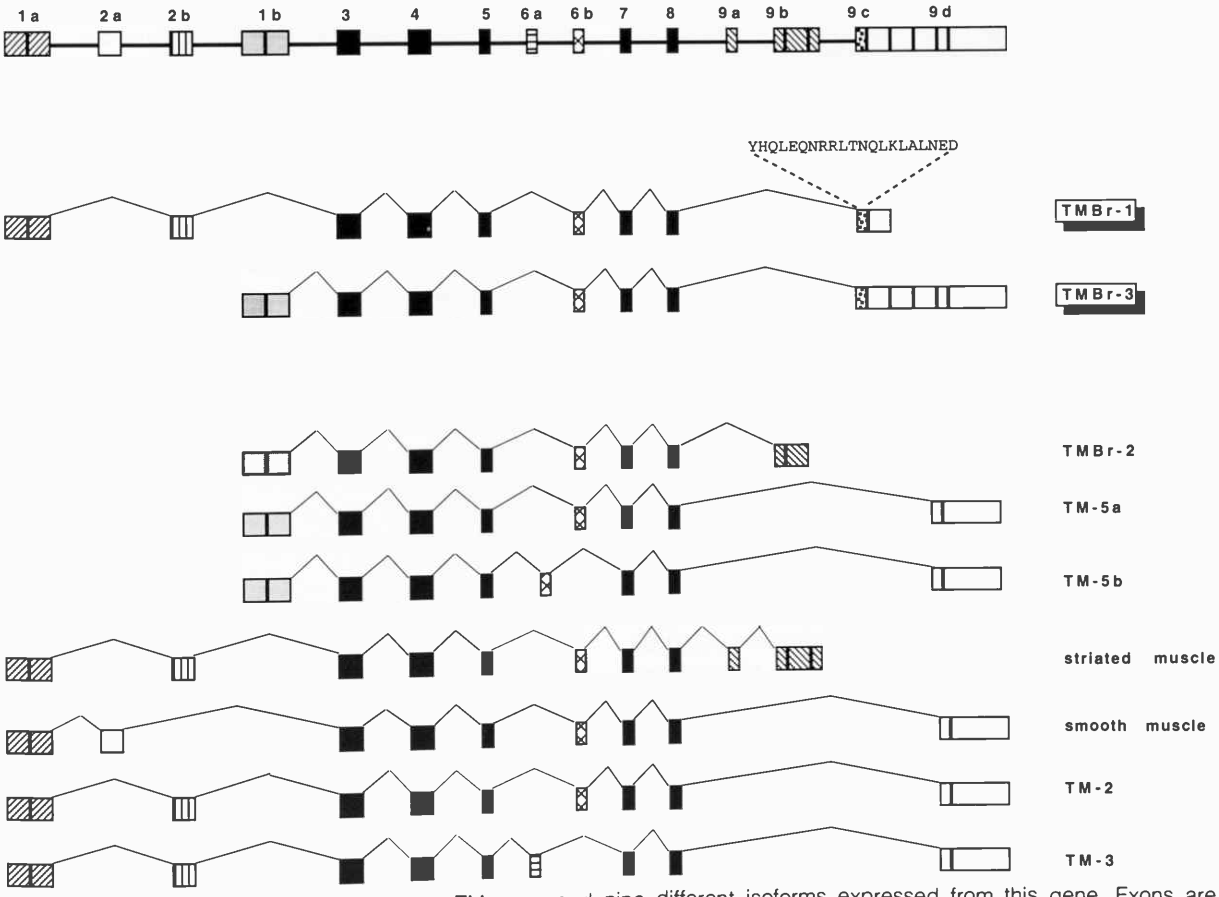

FIGURE 1 Schematic diagram of the rat α-TM gene and nine different isoforms expressed from this gene. Exons are represented as boxes and introns are represented as lines; they are not drawn to scale. The gene contains two alternative promoters that result in expression of two different amino-terminal coding regions (exons 1a and 1b), two internal mutually exclusive exon cassettes (exons 2a and 2b and 6a and 6b), and four alternatively spliced 3′ exons that encode four different carboxy-terminal coding regions (exons 9a, 9b, 9c, and 9d). Exons 3–5, 7, and 8 are common to all tropomyosin isoforms and are black. All other exons are alternatively spliced. The peptide that was used to raise a rabbit polyclonal antibody (anti-rTM9c) is indicated above exon 9c in the TMBr-1 mRNA.

identified factors in extracts derived from brain that bind specifically to sequences contained with the neuron-specific exon EN. Work is in progress to characterize these factors.

Since exon 9c of the α-TM gene is used only in brain and shares sequence identity with exon EN of the clathrin light chain B gene, we analyzed the developmental regulation of 9c using an exon-specific polyclonal antibody (see next section). Exon 9c is expressed at embryonic day E16 in the rat brain, at the same day when exon EN of clathrin light chain B is first expressed, and like exon EN, the expression of exon 9c is increased in the postnatal brain, reaching the highest level of expression in the adult brain. We are now trying to determine whether the regulation of exons EN and 9c involve common factors.

Brain-specific Tropomyosin Isoforms TMBr-1 and TMBr-3 Have Distinct Patterns of Expression during Development

S. Stamm, J. Lees-Miller, D. Helfman [in collaboration with Diana Casper, Mount Sinai School of Medicine]

Neurons differ significantly from other cell types in size and geometry. The morphology of neurons is essential to their function as conductors of information in a mostly vectorial manner. Their unique size and shape are reflected in the neuronal cytoskeleton. The polarity of neurons, related to the directional flow of information, has been manifested in a unique set of structural proteins creating this polarity, such as neurofilaments and microtubules with their associated proteins that can be preferentially segregated into axons or dendrites. We previously reported that the rat α-TM gene expresses at least nine distinct isoforms (see Fig. 1). Interestingly, the α-TM gene was found to express a unique set of isoforms in the rat brain, which were named TMBr-1, TMBr-2, and TMBr-3, and are 281, 251, and 245 amino acids in length with apparent molecular weights on SDS-PAGE of 36,000, 31,000, and 31,000, respectively (see also Fig. 1). We have generated an isoform-specific antibody specific for sequences contained within exon 9c of the α-TM gene and analyzed the developmental expression and regional distribution of these proteins in the developing and adult rat brain. Western blot analysis revealed that the two isoforms are differentially expressed in development, with TMBr-3 appearing in the embryonic brain at 16 days of gestation, followed by the expression of TMBr-1 at 20 days after birth (Fig. 2). In addition, although TMBr-3 was detected by Western blot in all brain regions examined (brainstem, cerebellum, cortex, hippocampus, midbrain, olfactory bulb, striatum, and thalamus), TMBr-1 was detected predominantly in brain areas that derive from the telencephalon (cortex, hippocampus, olfactory bulb, striatum, and thalamus). Immunocytochemical studies on mixed primary cultures made from rat embryonic midbrain indicate that expression of the brain-specific epitope is restricted to neurons, and this was confirmed by the lack of immunoreactivity when immunocytochemistry was performed on glial cultures of the same origin. The developmental pattern and neuronal localization of these forms of tropomyosin suggest that these isoforms have a specialized role in the development and plasticity of the nervous system.

At present, the functions of TMBr-1 and TMBr-3 are not known. The different developmental patterns and regional distributions of these isoforms suggest that these proteins will have different functions. Tropomyosins are known to be associated with filamentous actin in both muscle and nonmuscle cell types. Actin-based filaments are associated with a variety of neural structures, such as growth cones, dendritic spines, plasma membranes, axoplasm, presynaptic termini, and postsynaptic densities. They

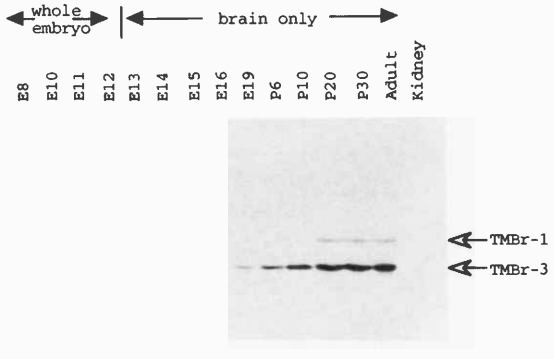

FIGURE 2 Expression of the brain-specific α-TM isoforms TMBr-1 and TMBr-3 at different developmental stages. Western blot analysis of equal amounts of protein (15 μg) using the rabbit polyclonal antibody, anti-rTM9c, that recognizes sequences encoded in exon 9c of the α-TM gene (see Fig. 1). Protein was prepared at E8–E12 from whole embryos and from whole brains at 13, 14, 15, 16, and 19 days of gestation (E13–E19) and 6–30 days after birth (P6–P30) and adult (3 month).

are thought to play a role in motile processes such as axoplasmic transport, growth cone movements, synaptic rearrangements, and vesicle movement. Work is in progress to further investigate the subcellular localization of these isoforms in neurons and brain tissue and to determine what proteins associate with these isoforms.

Functional Differences among Tropomyosin Isoforms

M. Pittenger, D. Helfman

Although the functions of tropomyosin in muscle tissue have been well studied, the roles of tropomyosin in nonmuscle cells are not understood. This is partly due to the large number of isoforms present and to the difficulty of separating the individual isoforms for study. We have been interested in elucidating the role(s) of the individual tropomyosin isoforms in cellular function. Utilizing cDNAs for each of the nonmuscle tropomyosins, we have used a bacterial expression system (pET system) to purify biochemical amounts of each tropomyosin for study. Tropomyosin proteins have few modifications and their relatively small size (29–36 kD) and stability make them ideal proteins for bacterial expression. These full-length, recombinant tropomyosins are not fusion proteins and contain only endogenous tropomyosin sequences. These tropomyosins have proven useful for in vitro experiments as well as introduction into cells by microinjection. We have shown in competition experiments that a 248-amino-acid isoform (TM-5b) can displace longer 284-amino-acid isoforms (TM-2 or TM-3) from the actin filament. These isoforms were each fluorescently labeled and microinjected back into cultured fibroblasts in order to ascertain their subcellular localization (Fig. 3). At the light microscope level, each was found to incorporate along microfilaments. Essentially identical subcellular distributions were observed for each isoform examined. It is worth noting that our experiments to date have been limited to fixed interphase cells and to α-TM gene products (TM-2, TM-3, TM-5a, and TM-5b; Fig. 1). We are in the process of extending these studies to include TM-1 and TM-4. In addition, we plan to examine the dynamic distribution of the different isoforms in living cells using video intensification microscopy during cell spreading, mitosis, and cell movement.

Although the bacterial expression system produces abundant and quite useful vertebrate tropomyosins, the bacteria do not acetylate the amino terminus, a modification common in eukaryotic cells. Due to head-to-tail overlap requirements between certain tropomyosins that would be adjacent along the actin filament, this acetylation can possibly effect the assembly of tropomyosins on filaments. To address such questions, we are using a baculovirus-derived expression system that will produce acetylated ver sions of tropomyosins. We have demonstrated that the TM-1 made in the baculovirus expression system is acetylated and shows enhanced F-actin binding compared to the same isoform produced in bacteria. We have also shown that the association of TM-1 binding to F-actin is greatly enhanced when an additional tropomyosin (TM-2) is also present. We are currently investigating the effects of the tropomyosin isoforms on the association of other microfilament-binding proteins, in particular, caldesmon, a protein whose association with microfilaments may be regulated by calcium/calmodulin signals. These studies will provide important information on the relationships between tropomyosins and the in vivo organization of microfilaments.

Partial Reversion of the Transformed Phenotype by Stable Expression of Specific Tropomyosin Isoforms in Transformed Cells

J. Kazzaz, M. Pittenger, D. Helfman

Transformation of cells results in a variety of cellular changes, including alterations in cell growth, adhesiveness, morphology, and organization of the cytoskeleton. Morphological and cytoskeletal changes are perhaps the most readily apparent feature of transformed cells. Although a number of studies have documented a decrease in the expression of specific tropomyosin isoforms in transformed cells, it remains to be determined if the suppression of tropomyosin synthesis is essential for the establishment and maintenance of the transformed phenotype. As a model system, we are using a Kirsten-virus-transformed cell line (ATCC NRK 1569). In contrast to normal fibroblasts, the Kirsten transformants contain reduced levels of TM-1 and undetectable levels of TM-2 and TM-3. These cells have a rounded morphology and are devoid of microfilament bundles.

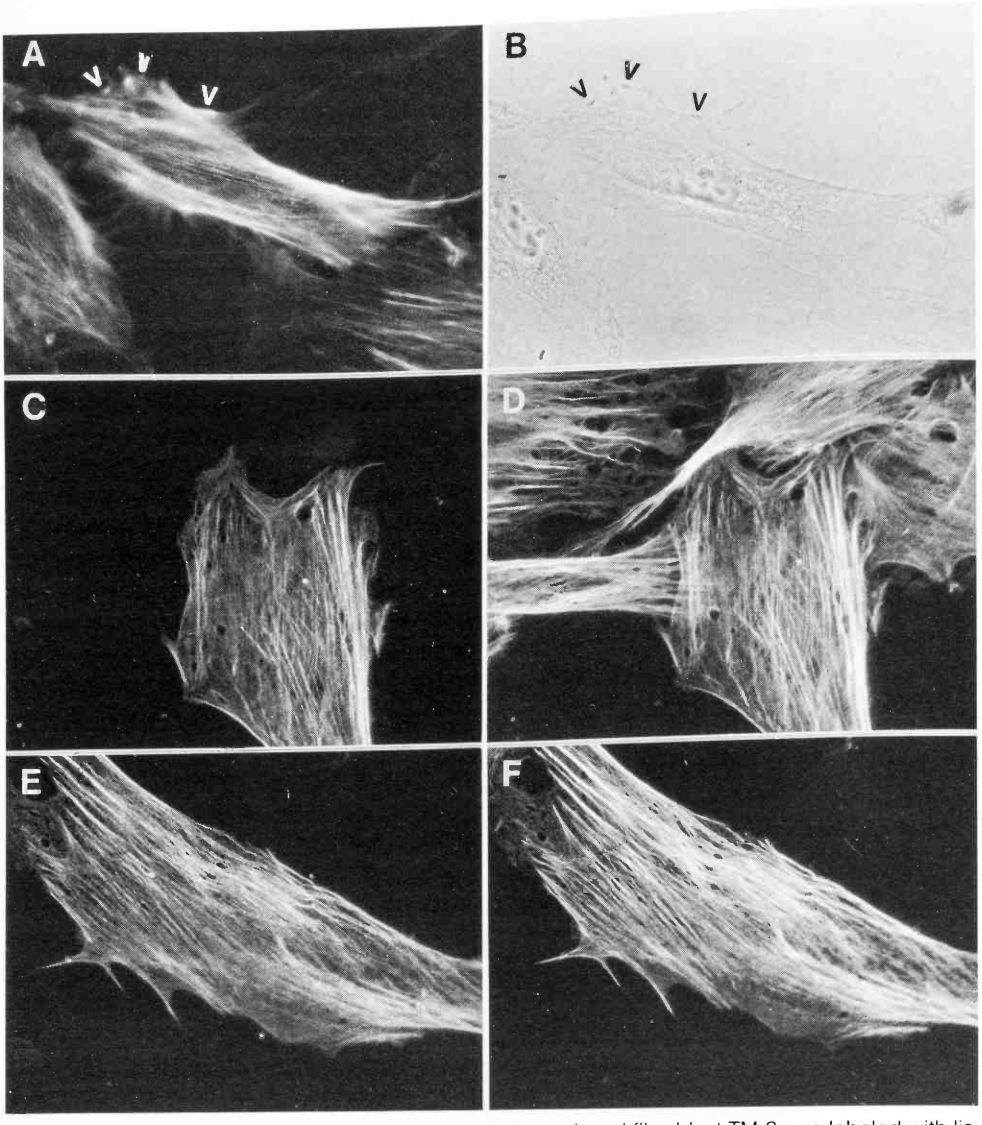

FIGURE 3 Localization of TM-3 in fibroblasts. Bacterially produced fibroblast TM-3 was labeled with lissamine rhodamine B sulfonyl chloride (LRB) and microinjected into rat fibroblasts (REF52 cells). (*A*, *C*, *E*) Examples of REF52 cells microinjected with LRB-TM3; (*B*, *D*, *F*) corresponding phase (*B*) or FITC-phalloidin-stained fields (*D*, *F*). TM-3 is distributed to the edge of the cell and in the edge ruffles (arrowheads). (*D*) Neighboring uninjected cells labeled with FITC-phalloidin.

We have prepared cDNA expression vectors for TM-2 and TM-3. Cell lines were established from the NRK 1569 cells that stably express TM-2 or TM-3. We have found that increased expression of these isoforms leads to cell spreading or flattening and formation of identifiable microfilaments. Other phenotypes include multinucleation and an increase in vacuoles and microspikes depending on the amount of tropomyosin expressed.

We are currently testing the cellular effects of co-expressing both TM-2 and TM-3, as well as comparing other characteristics of the transformed phenotype. Finally, we are introducing TM-1 into a adenovirus-transformed cell line that expresses no detectable amount of this isoform. In this way, we hope to delineate the functional significance of the altered pattern of tropomyosin synthesis in transformed cells.

A New Tropomyosin Essential for Cytokinesis in the Fission Yeast *Schizosaccharomyces pombe*

D. Helfman [in collaboration with M. Balasubramanian and S. Hemmingsen, Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan, Canada]

Mutations in the *Schizosaccharomyces pombe cdc8* gene impair cytokinesis. We have cloned *cdc8*$^+$ and found that it encodes a novel tropomyosin. Gene disruption results in lethal arrest of the cell cycle, but spore germination, cell growth, DNA replication, and mitosis are all unaffected. Haploid *cdc8* gene disruptants are rescued by expression of rat fibroblast TM-2. Immunofluorescence microscopy of wild-type and *cdc8* gene disruptants indicates that *cdc8* tropomyosin is present in two distinct cellular distributions: in dispersed patches and during cytokinesis as a transient medial band. Collectively, these results indicate that *cdc8* tropomyosin has a specialized role, which, we suggest, is to form part of the F-actin contractile ring at cytokinesis. These results establish the basis for further genetic studies of cytokinesis and of contractile protein function in *S. pombe*.

We have also found that rat fibroblast TM-3 can also complement the *cdc8* disruption in yeast, but two other fibroblast isoforms (TM-5a and TM-5b) are unable to complement the disruption. These results strongly suggest that the different fibroblast isoforms will have distinct cellular functions. As described in the preceding sections, experiments are in progress to study the role of the different isoforms in cell function.

A Vertebrate Actin-related Protein Is a Component of a Multisubunit Complex Involved in Microtubule-based Vesicle Motility

J.P. Lees-Miller, D. Helfman [in collaboration with T.A. Schroer, Department of Biology, Johns Hopkins University]

Actin is a cytoskeletal protein that is highly conserved across eukaryotic phyla. Actin filaments, in association with a family of myosin motor proteins, are required for cellular motile processes as diverse as vesicle transport, cell locomotion, and cytokinesis. Many organisms have several closely related actin isoforms. In addition to conventional actins, we and other investigators have found that yeasts (*Schizosaccharomyces pombe* and *Saccharomyces cerevisae*) contain actin-related proteins that are essential for viability. This year, we found that vertebrates also contain an actin-related protein (herein referred to as actin-RPV). Actin-RPV is a major component of the dynactin complex, an activator of dynein-driven vesicle movement, indicating that unlike conventional actins that work in conjunction with myosin motors, actin-RPV may be involved in cytoplasmic movements via a microtubule-based system. Interesting, actin-RPV (also known as centractin) was also found to be associated with centrosomes, where it may play a role in regulating microtubule-based motors required for centrosomal movements (Clark and Meyer, *Nature 359:* 246 [1992]).

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QUEST PROTEIN DATABASE CENTER

J.I. Garrels T.B. Boutell J. Horwitz
G.I. Latter J. Chien P.J. Monardo
B.R. Franza P. D'Andrea Z. Yu
S.D. Patterson

The QUEST Protein Database Center has been developing software and databases for analysis of two-dimensional (2D) gels. As a National Institutes of Health Biomedical Research Technology Resource, we have been preparing our 2D gel analysis software, now named Quest II, for distribution to the scientific community as a high-end tool for 2D gel protein database development. The Quest II software is aimed at users like ourselves, who wish to build quantitative databases from the analysis of 100 or more 2D gels, each containing 1000–2000 protein spots. The many improvements in the Quest II design, coupled with ever increasing computer power, at last promise to make 2D gel analysis nearly as rapid as 2D gel production. The Quest II system will be introduced to the user community through a Cold Spring Harbor Laboratory (CSHL) spring course in 1993.

Our database development work on human cells has progressed through the work of Scott Patterson and Robert Franza to identify key proteins and key protein modifications in lymphocytes. Jim Garrels has focused his database development work primarily on yeast, with the current emphasis placed on protein identification. He and Gerald Latter have made substantial progress on a new method of spot identification based on double-label amino acid analysis. These reports are presented in detail below.

One staffing change in 1992 was the addition of Tom Boutell as an applications programmer. He assists in the development of the Quest II system. The 2D Gel Laboratory Core Facility continues to be a key resource for our work (see Posttranslational Modifications and Apoptosis section in this Annual Report).

QUEST Software Project

P.J. Monardo, T.B. Boutell, J.I. Garrels, G.I. Latter

The Quest II software for analysis of 2D gels is now becoming available. We are in α test phase here at CSHL and are entering β test phase outside of the Laboratory. The software will be furnished to scientists who attend our first course on "Construction and Analysis of Two-dimensional Gel Protein Databases," a CSHL spring 1993 course.

An entirely new tool has been added to the system. This tool allows users to import their raw data into the system, to examine and linearize these data, and to merge multiple raw images, and smooth and detect them. The scientist can now perform these operations with no knowledge of computer commands.

Initial testing shows that the new system will greatly reduce the time to perform analysis of 2D gel experiments. These results can be stored as part of a permanent collection of experiments in a 2D gel protein database. Preliminary results also indicate

that our choice of a client/server architecture for the Quest II software may allow scientists to view each others' 2D gel data dynamically over the Internet, the national network linking major universities and scientific institutions.

CSHL is now in a position to become a center for providing 2D gel analysis software to other scientific institutions interested in constructing 2D gel databases. Our accumulation of experimental results and baseline data on the human lymphocyte work (see S. Patterson and B.R. Franza's reports in this section) will be put into a database that can be used by other scientists who may not want to repeat these experiments. Scientists can also send samples to the CSHL 2D gel core facility for comparison with other results in the database or for inclusion into the database. Our yeast and rat experiments will be converted for use with the new system. Keith Latham of the Fels Institute, our main β test site, will be putting his mouse embryo data into the new system.

For other organisms and cell lines, scientists at key institutions can use our software to acquire and store 2D gel data and linkages between the proteins on 2D gels and the genes that produced them.

Protein Identification by Double-label Amino Acid Analysis

J.I. Garrels, G.I. Latter

2D gel analysis is no longer limited by lack of computer hardware or software. However, it is severely limited by our lack of knowledge of the identities of the proteins we detect on 2D gels. We are attempting to achieve a general solution to this problem by accurate determination of partial amino acid compositions for the proteins resolved in our gels. Through biosynthetic labeling with two amino acids, one tagged with ^{14}C and the other with ^{35}S, we can determine the ratio of the two amino acids for each spot in the gel. Our computer modeling studies with yeast protein sequences, as reported last year, indicate that four ratios known to an accuracy of 3% should be adequate to identify nearly any protein from the yeast genome.

We have been applying the amino acid ratio method for both rat and yeast cells. Software has been developed to align images of double-labeled gels and to plot the rate of decay for each pixel in the gel accurately. We can determine through the rate of decay the ratio of the two amino acids in each pixel of the image. By averaging data for all the pixels in a spot, accuracy is substantially increased.

TABLE 1 Reproducibility of $^{14}C/^{35}S$ Isotopic Measurements for Related Spots

Protein	^{14}C Pro/^{35}S Met	^{14}C Leu/^{35}S Met
HSP73 form 1	0.227	0.192
HSP73 form 2	0.215	0.179
BIP form 1	0.311	0.204
BIP form 2	0.326	0.196
HSP90 form 1	0.109	0.166
HSP90 form 2	0.105	0.131
EF2 form 1	0.148	0.074
EF2 form 2	0.131	0.065
Vinculin form 1	0.130	0.089
Vinculin form 2	0.123	0.088

In a pilot experiment in rat cells, on which much of our prior database work has been based, we labeled WT2 cells with [^{14}C]proline and [^{35}S]methionine. In exposures taken over a 3-month period, we were able to determine ratios with an accuracy of 5%. In Table 1, we list some of the spots known or strongly suspected to be related by posttranslational modification. The results show that related spots have very reproducible $^{35}S/^{14}C$ ratios. This stage of the analysis shows us that the ratios are reproducible, and even one set of isotopic ratios allows us to find the unknown spot families that are likely to be related by posttranslational modification. This is especially easy to see when the spot image is displayed on the computer screen using color to depict the amino acid ratio.

The second step of our analysis leading to spot identifications is the conversion of isotopic ratios to amino acid ratios. We have selected eight protein spots of known amino acid sequence from the rat pattern, and we have plotted the known amino acid ratios versus the determined $^{14}C/^{35}S$ ratios. As shown in Figure 1, the data for known proteins generate a linear standard curve that can be used to determine the amino acid ratios for unknown spots. Approximately 500 spots from the gel are intense enough for determinations of the ratio of proline to methionine using this standard curve. This pilot experiment was far from optimal in several respects.

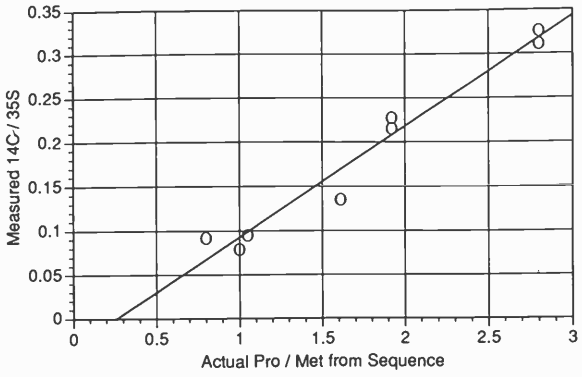

FIGURE 1 Standard curve for proline/methionine ratio determination in 2D gel spots. The measured $^{14}C/^{35}S$ ratio was determined for eight spots of known amino acid composition from a gel labeled with [^{14}C]leucine and [^{35}S]-methionine. When plotted against the known amino acid ratios, a linear standard curve was obtained. This curve can be used to obtain the amino acid ratios for unknown spots.

The overall incorporation of the two isotopes was not well-balanced, and the phosphorimager scans of the gel images were made at high sensitivity, which enhances the noise. Improved experiments have been conducted in yeast cells. Experiments using leucine versus methionine and leucine versus cysteine are now being analyzed. As expected, the ratio determinations have proved to be even more accurate, as measured by the reproducibility of determination for the pixels within a spot. Work is now progressing to identify enough yeast proteins to build the standard curve and to find optimal labeling conditions in yeast for linear incorporation of both amino acids.

We thank Dr. Dan Fraenkel of Harvard Medical School for making laboratory space available to Jim Garrels to conduct the yeast labeling experiments.

A Human 2D Gel Protein Database: "Baseline" Experiments and Posttranslational Modification Sets

S.D. Patterson, P. D'Andrea, J. Horwitz, Z. Yu

The human acute T-cell leukemic line, Jurkat (Clone E6-1), has been chosen for the initial experiments in the establishment of a human 2D gel protein database. This cell line is derived from an acute lymphoblastic leukemia, is used extensively in studies of cancer and AIDS, and closely resembles a normal stage IV peripheral T lymphoblast. A number of experiments considered to be "baseline" for the establishment of a more extensive database have been performed on these cells. The experiments include (1) an extensive protein turnover or time of labeling experiment with six different pulse times and three different chase periods, (2) three replicate heat-shock experiments, (3) PHA stimulation time course study, and (4) centrifugal elutriation (for cell cycle analysis).

These experiments are complete, with half of them being imaged using the old fluorography method, and half by phosphor autoradiography. Another important component of the human database will be the inclusion of sets for the identification of proteins carrying specific posttranslational modifications (Fig. 2). With one exception (Fig. 2f), these have all been performed using metabolic labelings with specific radioactive labels. The labelings (and labeling times) for which there are data on both whole-cell lysates and nucleus-enriched fractions for all listed include: (1) steady-state phosphorylation (3 hr and 8 hr), (2) myristylation (3 hr), (3) palmitoylation (3 hr), (4) prenylation (3 hr and 18 hr), (5) *N*-linked glycosylation, mannosylation (3 hr), and (6) *O*-linked glycosylation, labeling following lysis of the cells to detect carbohydrate chains with terminal *N*-acetyl-glucosamine using galactosyltransferase.

The mapping of which proteins in the methionine-labeled pattern carry these modifications is nearing completion. All of these radioactive labels, aside from phosphate, are tritiated. Therefore, these all have to be imaged for long periods of time using fluorographed gels (as tritium will not expose phosphorimaging plates). However, the use of [^{35}S]methionine/phosphate mixes to identify which proteins are labeled with ^{32}P has proven to be rapid using both film and phosphor autoradiography.

To identify members of the small GTP-binding protein or *ras* superfamily, the ability of these proteins to be renatured in situ on a nitrocellulose blot following 2D electrophoresis has been used to advantage. [^{35}S]Methionine-labeled cell lysates have been used to spike "cold" cell lysates for separation in a micropreparative mode (50 fg total protein). This allows sufficient protein to be blotted from the 2D gel to nitrocellulose for subsequent renaturation and incubation with [α-^{32}P]GTP. Many, but not all, small GTP-binding proteins are able to renature sufficiently to bind the labeled GTP. Approximately 20 proteins have been found to bind GTP using this method and through either quenching or not of the β-emission, ^{35}S-labeled proteins that bind GTP have been located.

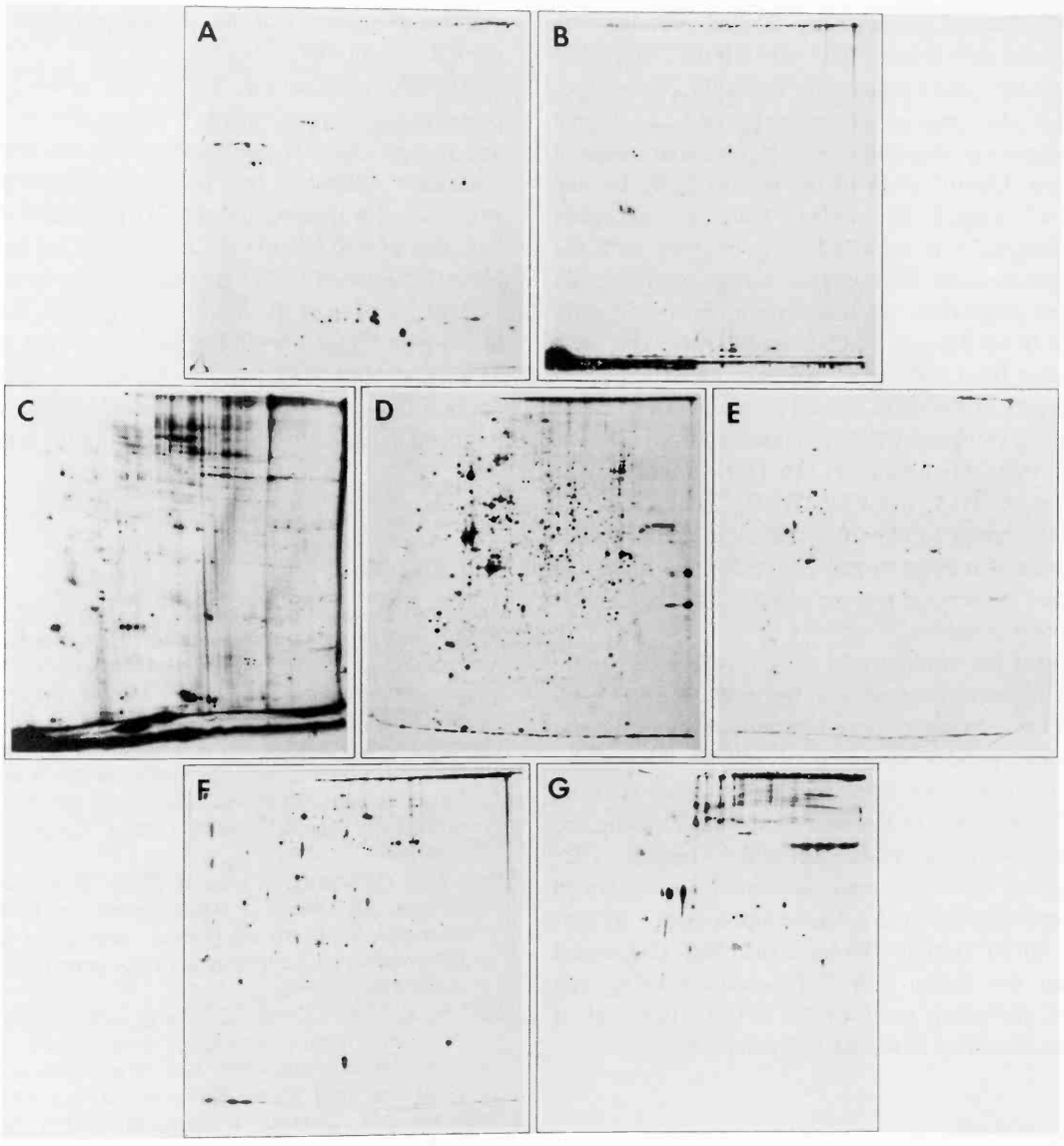

FIGURE 2 Composite of 2D gel images of whole-cell lysates (except G, which is from a nucleus enriched fraction) from posttranslational modification sets that form part of the human 2D gel protein database. The labels are as follows, with the label and labeling period in parentheses: (*A*) Prenylation ([^{3}H]mevalonate/3 hr); (*B*) myristylation ([^{3}H]myristate/3 hr); (*C*) phosphorylation ([^{32}P]orthophosphate/8 hr); (*D*) total protein ([^{35}S]methionine/30 min); (*E*) carboxylmethylation ([^{3}H]methyl-methionine/2 hr); (*F*) *N*-linked sugar ([^{3}H]mannose/3 hr); (*G*) *O*- linked sugar (post-lysis, nucleus-enriched, [^{3}H]galactose/galactosyltransferase).

Identification of Proteins in the Human 2D Gel Protein Database and the Initial Link to the REF52 Database

S.D. Patterson, P. D'Andrea, J. Horwitz

In addition to amino acid sequence analysis of proteins of specific interest (β-tubulin, during establishment of our protein chemistry methods, and numatrin [B23] for our apoptosis studies) and in vitro transcription/translation of full-length cDNA (see Posttranslational Modifications and Apoptosis in this section), we have begun to use enhanced chemiluminescence methods to identify proteins of interest in our 2D gel patterns. We are using the micropreparative system in the CSHL 2D Gel Laboratory Core Facility to separate 45 fg of cold protein spiked with

500,000 dpm of radioactively labeled protein, both from Jurkat cell lysates. The gels are then blotted to nitrocellulose and probed with antibodies (monospecific for the protein of interest), and the bound antibodies are visualized using chemiluminescent detection. Identification of the radioactively labeled proteins imaged by either film or phosphor autoradiography is achieved by overlaying with the chemiluminescent film image. Using commercially obtained antibodies, we have confirmed the identity of p42 MAP kinase, PLCγ1, and c-*raf1*, and with antibodies from colleagues, we have identified actin-RPV (vertebrate actin-related protein, Drs. Lees-Miller and Helfman, CSHL), numatrin (B23) (Dr. Olson, UMMC, Michigan), PCNA (Mr. Brand and Dr. Mathews, CSHL), and PTP-1B (Dr. Tonks, CSHL). We will attempt to identify other signal transduction molecules of interest to many investigators including ourselves to increase the usefulness of the human 2D gel protein database.

Another important aspect of this work is the desire to link this new database with the model REF52 database. This is being achieved initially through the use of limited peptide mapping of [^{35}S]methionine-labeled protein spots from both Jurkat and WT2 (a REF52 derivative in the REF52 database) using the reagent *N*-chlorosuccinimide, which cleaves after tryptophan. The fragments generated are separated using one-demensional gel electrophoresis in a Tris-tricine buffer system. We have matched 32 proteins between the Jurkat and WT2 patterns using this method, including some of the "PCNA-like" set of proteins identified in the REF52 database.

Human Regulatory Protein Sets

B.R. Franza [in collaboration with Rodrigo Bravo, Bristol Myers-Squibb, Jim Roberts, Fred Hutchinson Cancer Center, and Guilio Draetta, European Molecular Biology Laboratory]

We have identified cyclin E and D2 in H9 and Jurkat cells, and cyclin E has been identified in B lymphoblasts. We have characterized the cdc2 kinase family member, cdk2 in Jurkat cells, and are in the process of characterizing cdk3 in the same cells. In studies of cell cycle regulation, we have identified a protein tyrosine phosphatase in human lymphoblasts and have shown that its phosphorylation state is altered through the cell cycle.

The characterization of Rel and related cellular proteins has been extended. We have shown that Rel and the p50-NF-κB precursor, p105, are complexed with each other and that both are phosphoproteins. We have also determined the 2D gel position of the isoforms of p50 NF-κB itself and think we have p65-NF-κB identified in 2D gel images. We have identified the location of the inhibitor of NF-κB, IκB (p40) in 2D gels. Thus, our ability to monitor the behavior of several classes of regulatory proteins using databases constructed from 2D images of cellular proteins has been expanded significantly during the past year.

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GENETICS

There was an old man from Bombay
who was asked what he thought of DNA
He said: "Could be worse,
I might have the curse,
of purifying proteins all day."

Genetics is the study of organisms from the perspective of their inherited traits. From the days of Mendel's genetic crosses with sweet peas in a monastery in Central Europe, to contemporary studies at Cold Spring Harbor, the underlying logic of the art of genetics has remained largely unaltered. However, we now use a battery of methods including gene cloning, whole-genome approaches, two-hybrid yeast screens, and differential library screens and many more techniques that enormously expand the power and scope of classical techniques. As in past years, we continue to use a range of model organisms from yeast to plants. The most recent revolution, one which will have widespread implications, is the application of new methods to the study of human genetics. The time-tested model organisms such as yeast are still needed, but increasingly human disease and their genes can be studied directly. The work of the genetics group is described below.

EUKARYOTIC CELL CYCLE CONTROL

D. Beach

S. Allan
M. Caligiuri
D. Casso
T. Connolly
S. Davey
D.J. Demetrick
H. Feilotter
K. Galaktionov
I. Garkavtsev
C. Gawel
G. Hannon
J. Hofmann
J. Hudson
N. Kaplan
D. Lombardi
K. Lundgren
S. Matsumoto
T. Matsumoto
B. Nefsky
K. Okamoto
M. Serrano
A. Tesoro
N. Walworth
Y. Xiong
H. Zhang

During the course of the year, Jim Bischoff left to take a position with Onyx Pharmaceuticals; Lisa Molz obtained a Ph.D. and moved to Rusty Williams' laboratory at the University of California at San Francisco; Guillaume Cottarell joined Mitotix; Toru Mizukami returned to Kyowa Hakko Kogyo Co., Ltd., Japan; and Catherine Jessus returned to CNRS in France. We were joined by Greg Hannon, Jim Hudson, Manuel Serrano, and Sanae Matsumoto.

Cellular Transformation and Cyclin-CDK Complexes

Y. Xiong, H. Zhang, D. Beach

Following the isolation of the human D-type cyclin gene family, we have focused our efforts on identifying cyclin-D-associated catalytic subunits and other cellular proteins that interact with D cyclins. In hu-

man diploid fibroblasts, we found that cyclin D1 is complexed with many other cellular proteins. Among them are protein kinase catalytic subunits cdc2, cdk2, cdk4 (previously called PSK-J3), and cdk5 (also called PSSALRE). In addition, polypeptides of 21 kD and 36 kD were identified in association with cyclin D1. We showed that the 36-kD protein is the proliferating cell nuclear antigen (PCNA). Cyclin D3 also associates with multiple protein kinases, p21, and PCNA. It was proposed that there exists a quaternary complex of D cyclin, cdk, PCNA, and p21 and that many combinatorial variations (cyclins D1, D3, and cdk2, 4, and 5) may assemble in vivo. These findings link a human putative G_1 cyclin that is associated with oncogenesis with a well-characterized DNA replication and repair factor.

Subsequent to the identification of the cyclin-D quaternary complex, we discovered that PCNA and p21 are present in all cyclin-cdk complexes that we have examined as universal elements in normal human fibroblasts. Upon transformation of diploid fibroblasts with the DNA tumor virus SV40 or its transforming tumor antigen (T), the quaternary cyclin-cdk complexes undergo subunit rearrangements. The cyclin D-p21-cdk-PCNA complexes are disrupted. In transformed cells, CDK4 totally dissociates from cyclin D and is replaced by p16. Quaternary complexes containing cyclin A-p21-cdk-PCNA are rearranged in the transformed cells. A polypeptide of 19 kD was identified in association with cyclin A and only appears in the transformed cells. Both p21 and PCNA are dissociated from cyclin B1-cdk complexes. The pattern of subunit rearrangement of cyclin-cdk complexes in SV40-transformed cells is also shared in those containing adenovirus and papillomavirus oncoproteins. These findings provide the first evidence for the alteration of protein complexes that are involved in cell cycle control during cellular transformation.

Cell Cycle Genes in Human Neoplasia

D.J. Demetrick, D. Beach

During the past year, our laboratory has attempted to define the potential role of cell cycle regulatory gene products in human neoplasia. Genetic alterations of a variety of cellular genes have been demonstrated to contribute to human cancer. Some of these genetic alterations have been associated with a chromosomal translocation that either can result in fusion products with other genes, conferring abnormal protein function (chronic myelogenous leukemia t[9:22]), or can result in a change in the regulation of expression of a tightly regulated gene (Burkitt's lymphoma t[11:14]). Since the expression of many cell cycle genes appears to be highly controlled, it is possible that alterations in their normal regulation will lead to the abnormal cell proliferation present in most human neoplasia. A cell cycle gene identified by our laboratory, among others (cyclin D1), has already been shown to most likely encode the elusive *bcl-1* oncogene. Our strategy has been to map the newly identified human cell cycle regulatory genes cloned by our laboratory to their chromosomal locations and to study their expression in tumors and cell lines that show gross genomic abnormalities involving chromosome locations near those genes. This year, the human cell cycle phosphatase cdc25A was mapped by our laboratory to chromosomal position 3p21 and cdc25B was mapped to 20p13 utilizing fluorescence in situ hybridization techniques and the polymerase chain reaction. Some histological subtypes of lung and renal carcinomas show common karyotypic abnormalities involving 3p21. The tissue-specific patterns and normal levels of expression of cdc25A and cdc25B have been determined and their expression in tumors demonstrating abnormal karyotypes near the loci of those genes is currently being investigated. Our laboratory is also currently mapping a variety of other cell cycle regulatory genes to help determine whether these genes are involved in the genetic alterations observed in the cells of some human tumors.

Rbr-2, an Rb-related Protein, Interacts with the cdk2 Kinase

G. Hannon, D. Beach

To identify proteins that might play key roles in controlling transit through the G_1 and S phases of the mammalian cell cycle, we sought to isolate previously unknown proteins that could interact with a known cell cycle regulatory kinase, cdk2. Toward this goal, we have used a "two-hybrid" protein interaction screening procedure. Previously, the discovery of an interaction between two proteins required not only substantial information about each potentially inter-

acting member, but also a direct test for their interaction. The two-hybrid system developed by S. Fields (SUNY, Stony Brook) and colleagues overcomes this limitation.

We constructed a cDNA library (derived from HeLa cell mRNA) that directs the expression of human proteins as fusions with the yeast GAL4 transcriptional activation domain. In addition, we constructed a vector that directs the expression of the cdk2 protein kinase as a fusion with the GAL4 DNA-binding domain. These were cotransformed into a yeast strain that carries the *his3* and *lacZ* genes under the control of GAL4-dependent promoters. If an individual cDNA directs the synthesis of a fusion protein that can bind to cdk2, then a competent transcriptional activator is formed, and the *his3* and *lacZ* genes are expressed. Selection for these gene products can be used to identify such cDNAs, and to date, three have been isolated by using this procedure.

One cDNA encoded a human homolog of the fission yeast Suc1 protein. Suc1 is a protein of unknown function that is known to tightly bind the fission yeast cdc2 protein. The human Suc1 homolog had been previously isolated and was known to interact with cdk2 physically. Thus, the isolation of the human Suc1 homolog provided confirmation that the two-hybrid screen was capable of identifying proteins that could interact with cdk2.

A second cDNA encoded a previously unknown protein with a predicted molecular mass of 24 kD. This protein showed no homology with any protein present in the currently available databases, and experiments to ascertain the function of this protein are currently under way.

The third cDNA encoded a 1082-amino-acid protein whose sequence showed striking similarity to a class of proteins, the nuclear "pocket" proteins, of which the tumor suppressor protein, Rb, is the founding member. The protein encoded by this cDNA has been termed Rbr-2 (Rb-related) based on this homology.

The sequence of Rbr-2 was most similar to the sequence of another Rb-related protein known as p107. Rbr-2 shows approximately 50% amino acid identity with p107 over the entire length of available p107 sequence. Homology with Rb is restricted to the A and B subdomains, which comprise the "pocket" domain, and to the "amino-terminal homology region," which is also shared between Rb and p107.

The pocket domains of Rb and p107 are known to bind to proteins encoded by DNA tumor viruses: SV40 T antigen and adenovirus E1A. We have found that this property is also shared by the pocket domain of Rbr-2. We are currently investigating the possible interaction of Rbr-2 with known cell cycle regulatory proteins. In particular, we are testing whether the interaction between Rbr-2 and cdk2 indicated by the initial yeast screen occurs through a direct physical interaction or, alternatively, through interaction of both of these proteins with a cyclin. In this regard, p107 is known to bind cyclin A.

The interaction between Rbr-2 and cdk2 (whether direct or indirect) provides another example of the cross-talk between the basic cell cycle regulatory machinery and the nuclear "pocket" proteins. By binding to transcription factors, the "pocket" proteins are thought to regulate the expression of a series of genes that are critical for cell growth. Thus, interaction between cdk2 and Rbr-2 may provide a mechanism for the control of gene expression by cyclin-cdk complexes.

Regulation of $p34^{cdc2}$ and Cell Cycle Arrest in Response to DNA Damage

N. Walworth, S. Davey, D. Beach

The regulation of the activity of $p34^{cdc2}$ in fission yeast is dependent on both complex formation of $p34^{cdc2}$ with the product of *cdc13* and phosphorylation of $p34^{cdc2}$ on threonine and tyrosine residues. Tyrosine phosphorylation of $p34^{cdc2}$ prevents entry into mitosis and is dependent on the products of the *mik1* and *wee1* genes. Simultaneous loss of function of *mik1* and *wee1* results in premature advancement into mitosis leading to cell death. Cold-sensitive alleles of *cdc2* were found that permit the cell to survive without functional *mik1* and *wee1*, but themselves lead to a cell division cycle defect at low temperature. Multicopy expression of *cdc13*, a B-type cyclin that is known to associate with $p34^{cdc2}$, suppresses the cold-sensitive growth defect of these *cdc2* alleles. A genomic library prepared from *Schizosaccharomyces pombe* DNA has been introduced into two of these cdc2 strains, and high-copy suppressors have been identified. One of these suppressors encodes a novel protein kinase, chk1. This kinase is dispensable for vegetative growth; however, loss of function of chk1 causes cells to be sensitive to both UV radiation and incompletely replicated DNA. Characterization of *chk1* suggests that it is required

for the checkpoint that temporarily arrests the cell cycle in response to DNA damage; *chk1* encodes a *ch*eckpoint *k*inase.

"Checkpoint" is a term used to describe systems used by the cell to ensure that downstream events do not take place unless upstream events have been properly completed. Perhaps the best studied example is the dependence of mitosis on the completion of DNA replication. Similarly, cells must ensure that mitosis is not initiated if DNA has been damaged. For example, when wild-type cells are exposed to UV light, they normally arrest the cell cycle in G_2 in order to repair the damage. The absence of *chk1* does not impair the cell's ability to repair damaged DNA; rather, the cell fails to recognize that DNA damage has occurred and attempts to undergo mitosis with damaged chromosomes. The phenotype of *chk1* is similar to that of a subset of mutants that were isolated because they are sensitive to radiation (*rad* mutants). Four of the *rad* mutants, including *rad1-1*, are also defective in the checkpoint which signals to the cell that DNA damage has occurred. Multicopy expression of *chk1* partially suppresses the UV sensitivity of *rad1-1*. Therefore, *chk1* may provide a link between the *rad*-dependent DNA-damage-sensing pathway and *cdc2*-dependent control of the cell cycle.

Regulation of the G_1/S Phase Transition in Fission Yeast

M. Caligiuri, D. Beach

In *S. pombe* as in other eukaryotic organisms, commitment to the mitotic cell cycle occurs in G_1 at START. Only two genes whose products are required for the passage through START in fission yeast have been identified to date. One is *cdc2*, which is also required in G_2, and the other is *cdc10*. Strains arrested in G_1 at either the *cdc10* or *cdc2* restriction points will conjugate under appropriate nutritional conditions, indicating that cells are not committed to the mitotic cell cycle until after the execution of $p34^{cdc2}$ and $p85^{cdc10}$ functions. $p85^{cdc10}$ shares amino acid sequence similarity with the Swi4 and Swi6 transcription factors from *Saccharomyces cerevisiae* and has been shown to be a component of a DNA-binding complex that recognizes the promoters of the *cdc22* and *cdt1* genes.

We have investigated the role of *cdc10* in the regulation of the G_1/S-phase transition through the characterization of the *sct1-b1* gene, a dominant extragenic suppressor of a temperature-sensitive *cdc10* mutant. The *sct1-b1* gene suppresses several conditional alleles of *cdc10* as well as a *cdc10* disruption. $sct1^+$ encodes a protein of 637 residues with 36% amino acid sequence similarity to $p85^{cdc10}$. The sct1 polypeptide also shares amino acid sequence similarity with the Swi4 and Swi6 polypeptides which spans a region containing two 33-amino-acid repeats. These repeats have been identified in a diverse group of proteins and have been implicated in providing dimerization interfaces in heteromeric protein complexes. The similarity between sct1 and Swi4 is not limited to the region containing these repeats; sct1 also shares striking similarity with the DNA-binding domain of Swi4. Through a comparison of the mutant *sct1-b1* and wild-type $sct1^+$ genes, we have identified a single amino acid substitution that lies in the putative DNA-binding domain. This mutation renders the cell independent of the 33-amino-acid repeats and of *cdc10* for the execution of START.

We have shown that the *sct1* gene product, $p72^{sct1}$, binds to the promoters of the *cdc22* and *cdt1* genes in association with $p85^{cdc10}$. Characterization of the *sct1* null allele has indicated that *sct1* is an essential gene which functions at START. Cells arrested at the execution point do so as elongated cells that are capable of conjugation. In fact, cells carrying the *sct1* null allele conjugate even when grown on rich media, a condition that is inhibitory for mating between wild-type cells. These data suggest that *sct1* plays a pivotal role in the life cycle of *S. pombe* as a transcription factor that acts as a positive regulator of the mitotic cell cycle and a repressor of its alternative developmental fate leading to sexual differentiation.

Isolation of Genes Required for the G_1/S Transition

J.F.X. Hofmann, D. Beach

The *cdc10* gene product is required for cell cycle progression at the G_1/S boundary in fission yeast. Its function is executed immediately before the initiation of DNA synthesis under a variety of different physiological conditions. Recent findings suggest that *cdc10* functions as a transcription factor that is required for the cell-cycle-regulated expression of a

number of genes required for the entry into S phase. To identify these presumptive target genes, we prepared protein extracts from *S. pombe*, and these extracts were incubated with size-fractionated genomic yeast DNA. Using specific antibodies directed against the *cdc10* gene product, protein-DNA complexes were immunoprecipitated, and enriched genomic DNA was amplified by PCR. These hypothetical promoter sequences were used to screen gene banks to isolate the respective genes.

Three of the isolated genes have been found to be periodically expressed in the cell cycle, with a peak of their steady-state mRNA level in late G_1. Sequence analysis has established that one of the genes is identical to *cdc22*, which has been proposed recently to be regulated by *cdc10*. The two other genes have not been identified previously and have been termed *cdt1* and *cdt2*.

We have shown by DNA footprint analysis that a protein complex containing the *cdc10* gene product specifically binds to the *cdt1* promoter, suggesting that *cdc10* is directly involved in the transcriptional regulation of *cdt1*. The role of this binding site in the expression of *cdt1* is under investigation.

Gene disruption experiments have shown that *cdt1* is an essential gene and that it is required for the G_1/S transition. We have also shown that overexpression of *cdt1* from a heterologous promoter in cells that grow under rate-limiting conditions for the entry into S phase results in a transient acceleration of this event. Ectopic expression of *cdt1* can also complement a temperature-sensitive mutation of *cdc10* at semipermissive temperature.

These experiments suggest that the cell-cycle-regulated expression of *cdc10* and at least one other factor is dependent on *cdc10* and that their expression is rate limiting for the G_1/S transition. Further genetic screens are directed toward the identification of additional genes required for the G_1/S transition.

Construction of the Fission Yeast Physical Map

T. Mizukami, I. Garkavtsev, N. Kaplan, T. Matsumoto
D. Beach [in collaboration with T. Marr, W. Chang, and
D. Lombardi, Cold Spring Harbor Laboratory, and
M. Yanagida, O. Niwa, and A. Kounosu, Kyoto University]

For the mapping of the fission yeast genome (length about 14 Mb), a fivefold representative cosmid bank (1920 clones, primary bank) and phage library were prepared. At the beginning, four kinds of probes representing known repetitive sequences (centromeric repeats, 17S and 5S ribosomal repeats, and the long terminal repeat of transposon elements) were hybridized to the primary cosmid bank. T3/T7 probes were prepared from cosmid clones that were not hit by these probes. Cosmid clones were chosen randomly based on only one criterion—that the clones had not yet been hit. More than 600 of this type of hybridization experiment were done. As a result of our strategy, we were able to assemble clones into contigs. Simultaneously, contigs were anchored onto chromosomes. For this purpose, we used three kinds of physical landmarks: more than 50 markers with known genetic localization on chromosomes, about 70 new genes that were previously unmapped, and *Sfi*I and *Not*I restriction sites. A complete *Sfi*I restriction map of the *S. pombe* genome was constructed previously. Some of the contigs that had no genetic markers were located by pulse-gel electrophoresis. At the end of this strategy, the three chromosomes assembled into a total of 60 contigs. In the next step, real gaps between contigs were filled by probing a "deeper" cosmid bank (these cosmids were not included in the 1920 clones) and phage library. T7/T3 probing from the very end clones is still in process. We now have 12, 5, and 6 contigs on chromosomes 1, 2, and 3, respectively.

The hybridization mapping strategy is being conducted in parallel with *Pst*I/*Bam*HI restriction mapping of the genome. Restriction mapping using six-base-pair-recognized restriction enzymes enables us to determine the real physical map of the genome with good resolution in kilobase units. Approximately 70% of the cosmids from the primary bank were digested with *Pst*I, *Bam*HI, and *Pst*I/*Bam*HI. Because overlap information is available from the hybridization mapping, these restriction digests allow assembly of a unique *Bam*HI/*Pst*I map.

From the analysis of more than 2000 cosmids and phages, we will be able to designate a unique set of recombinant clones on one membrane that will represent sequences for virtually the entire *S. pombe* chromosomes. About 450 recombinant clones, which are termed the "miniset," represent almost all cloned fragments and split the whole genome into 450 segments. Each segment has specific chromosomal localization and can be used for determining specific locations for any genes or unknown fragments of DNA on the specific chromosomal sites. The miniset of clones was chosen for a global systematic genome sequencing project.

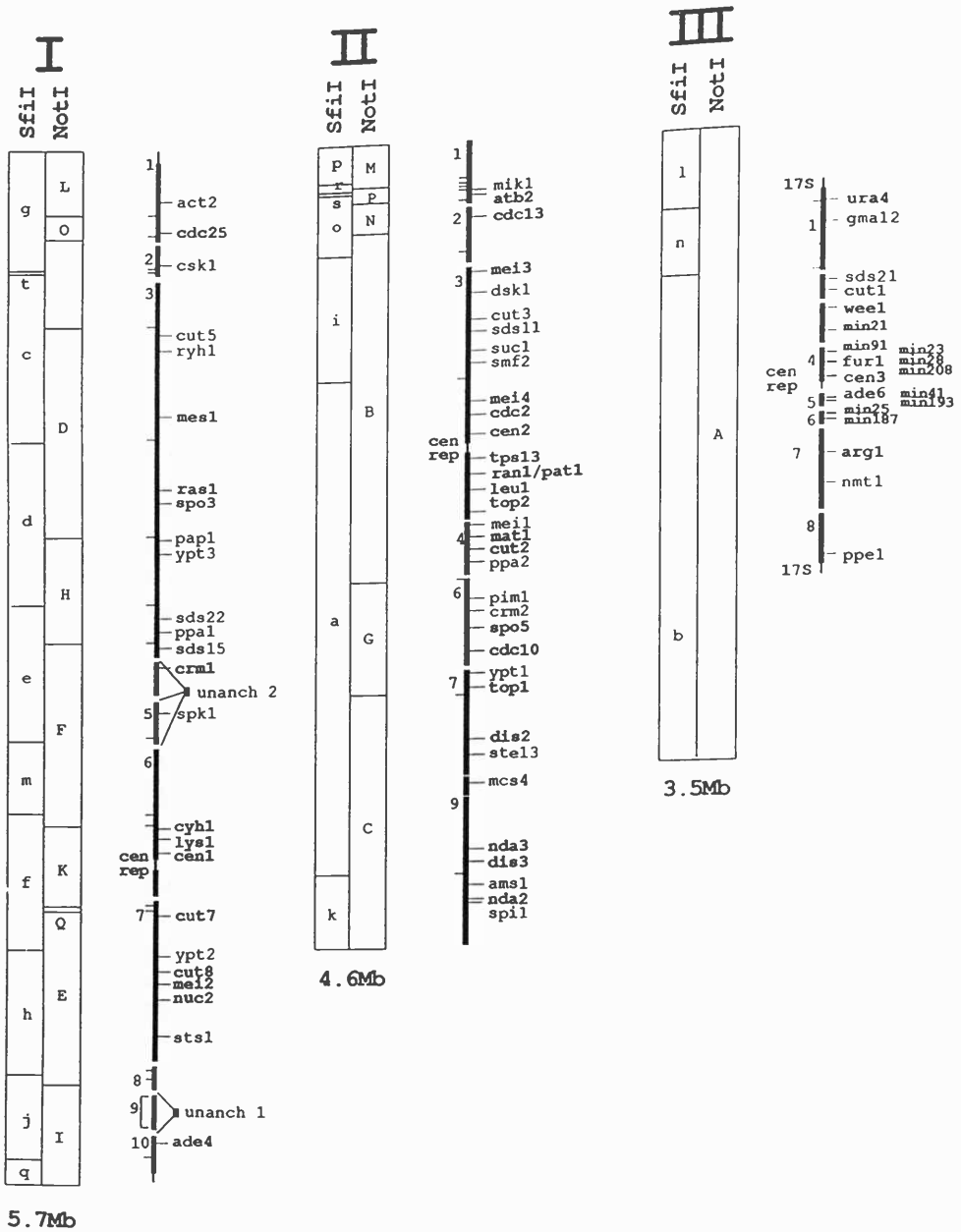

FIGURE 1 Contig map of the fission yeast contigs are placed to the right of the *Not*I and *Sfi*I restriction maps. The position and length of each contig reflect their localization on the chromosomes. Genetically mapped markers are indicated with bold letters, and new genes that were previously unmapped are shown with regular type. Thin lines on the left side of contigs indicate locations of *Sfi*I and *Not*I restriction sites.

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PLANT GENETICS

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V. Sundaresan A. Baron C.-D. Han P. Springer A. Yonetani
T. Peterson J. Colasanti J. Mystowski R. Srinivasan Z. Yuan
L. Das

An Enhancer-trap/Gene-trap Mutagenesis System in *Arabidopsis*

R. Martienssen, P. Springer, A. Baron, S. Haward (URP Program), J. Mystowski (Partner for the Future), T. Volpe, H. Ma, V. Sundaresan [in collaboration with C. Dean (Cambridge Laboratory) and J. Jones (Sainsbury Laboratory), John Innes Centre for Plant Science Research, Norwich, England]

Last year, we reported on the initiation of an enhancer-trap/gene-trap system for identifying and disrupting genes expressed at various stages of plant development. On the basis of similar systems in *Drosophila* and mouse genetics, we have developed a transgenic plant system for mobilizing transposable elements carrying an appropriate reporter gene around the *Arabidopsis* genome. This will allow the identification of target genes both by virtue of their pattern of expression and by virtue of any mutant phenotype caused by transposon insertion. We are using McClintock's *Ac/Ds* transposon system from maize, which has been shown to transpose at high rates in *Arabidopsis* when suitably modified for its new host (Bancroft et al., *Mol. Gen. Genet. 233:* 449 [1992]; Swinburne et al., *Plant Cell 4:* 583 [1992]. We have constructed two classes of reporter elements: (1) an enhancer-trap element, which has a minimal promoter fused to the reporter gene, and (2) a gene-trap element, which has a multiple splice acceptor fused to the reporter gene. As described last year, the β-*glucuronidase* (*GUS*) reporter gene in these constructs was shown to respond to the presence of external enhancers and promoters by transient expression experiments. Several stable transformants were obtained with each of these con-

structs, and also with an *Ac* transposase construct, obtained from J. Jones (Sainsbury Laboratory), which we modified for use with our selection scheme (described below).

When *Ds* elements excise in *Arabidopsis*, they frequently fail to reinsert, and when they do, it is typically close to their original location. Selection schemes based on transposon excision will therefore recover many lines that have no transposed elements or transposed elements that are concentrated in a small region of the genome. We are using a novel selection scheme that avoids these problems by selecting not for excision, but rather for unlinked transposition. This will maximize the recovery of independent randomly distibuted insertions. In our scheme, both the *Ds* and *Ac* constructs carry an adjacent counter-selectable marker gene that encodes indole-acetic acid hydrolase, an enzyme involved in auxin biosynthesis. When plants are grown on the nontoxic auxin precursor α-naphthalene acetamide, those that have the *iaaH* gene convert the precursor into naphthalene acetic acid (NAA), a powerful auxin. At toxic concentrations, NAA inhibits shoot growth and causes a proliferation of short roots, resulting in stunted seedlings. In addition, the *Ds* element carries a positive selectable marker, the neomycin phosphotransferase II gene, that confers resistance to kanamycin (Kan). Plants heterozygous for both *Ds* and *Ac* are generated by crossing parent *Ac* and *Ds* lines together. Transposition of *Ds* occurs in these heterozygotes and results in individual flowers that carry new transposed elements in their germ line. When these flowers set seed by self-pollination, the only progeny that are resistant to both Kan and NAM are those that carry a *Ds* element (which confers Kan resistance), but not the *Ds* donor locus (which confers NAM sensitivity). These progeny must carry an unlinked, or loosely linked, transposed element. Following the practice in *Drosophila*, they are referred to as transposants.

During the past year, the transgenic plants that we generated have been characterized genetically and molecularly. Five different lines have been established that are homozygous for single loci that carry the *Ac* transposase gene, driven by a strong promoter. These *Ac* lines have been crossed to *Ds* tester lines (provided by C. Dean) to confirm that they carry functional transposase. Eight transgenic "starter" lines have also been established that are homozygous for single loci carrying either *DsE* (the enhancer-trap

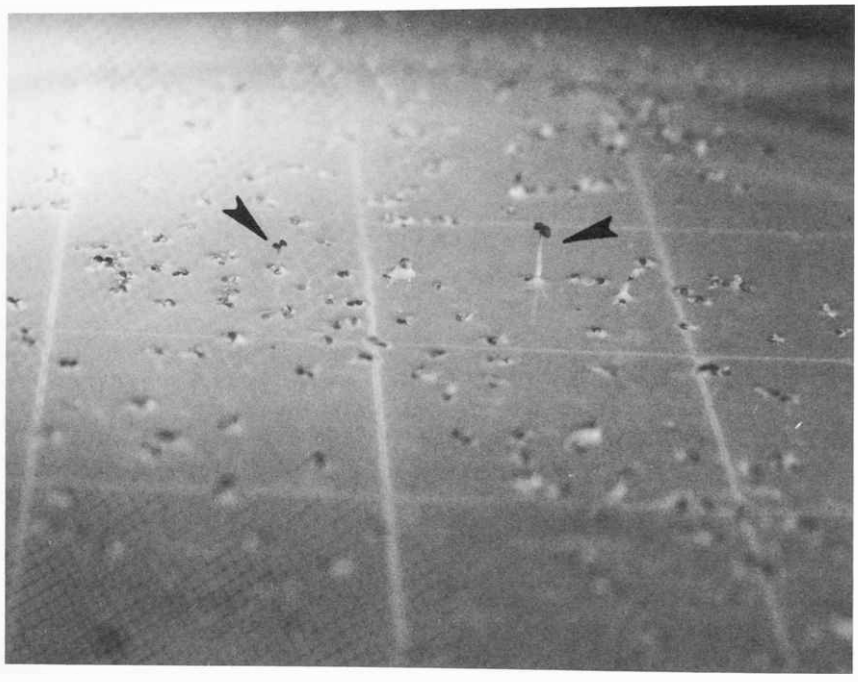

FIGURE 1 Selection for unlinked transpositions: F2 progeny from an *Arabidopsis* plant heterozygous for both a gene-trap *Ds* and *Ac* transposase were germinated on media containing NAM and Kan. Two seedlings carrying a transposed element are doubly resistant to NAM and Kan (arrows).

Ds) or *DsG* (the gene-trap *Ds*). The *Ac* and *DsE* line and the *Ac* and *DsG* line have been crossed together in order to generate 10,000 F1 seed. Eventually, all of these F1 progeny will be grown and self-pollinated to generate transposants in the F2 generation. Each transposant line will then be screened for patterns of reporter gene expression in the F3 generation. In a pilot study, we have allowed about 200 F1 plants to self-pollinate. F2 seed from these plants have been sown on selective media, and the selection conditions have been optimized to enable large-scale screening for transposants (Fig. 1). Seven out of 21 enhancer-trap F1 plants and 88 out of 218 gene-trap F1 plants gave some F2 progeny that survived double selection (i.e., transposants). In these families, transposants comprised between 0.1% and 5% of the progeny. DNA was isolated from 20 different transposants and analyzed by Southern blotting and PCR. As expected, these plants had *Ds* transposons at novel locations and did not carry the donor locus or the *Ac* transposase gene. A few of the *Ac*/+ *DsG*/+ F1 plants were stained for reporter gene activity, and one was found to have a large clonal sector comprising two adjacent leaves, in which the epidermal guard cells were deeply stained (Fig. 2). Two out of seven enhancer-trap F2 transposants also expressed the reporter gene at the seedling stage. One of them displayed constitutive expression, whereas the other displayed expression restricted to cotyledons and stipules. These results suggest that both the *DsG* element and the *DsE* element can transpose into genes and result in cell-type-specific expression of the reporter gene.

In the coming year, we hope to generate 1000–2000 transposant lines and screen them for patterns of reporter gene expression. On the basis of our preliminary screens, we hope to recover several hundred lines that have either a novel pattern of reporter gene expression or a transposon-linked mutant phenotype, or both. Our selection scheme makes generating large numbers of informative transposant lines both practical and efficient. It is a significant improvement over existing schemes based on selection for excisions and may prove to be essential when screens for reporter gene patterns involve embedding and sectioning plant tissue, or other laborious procedures.

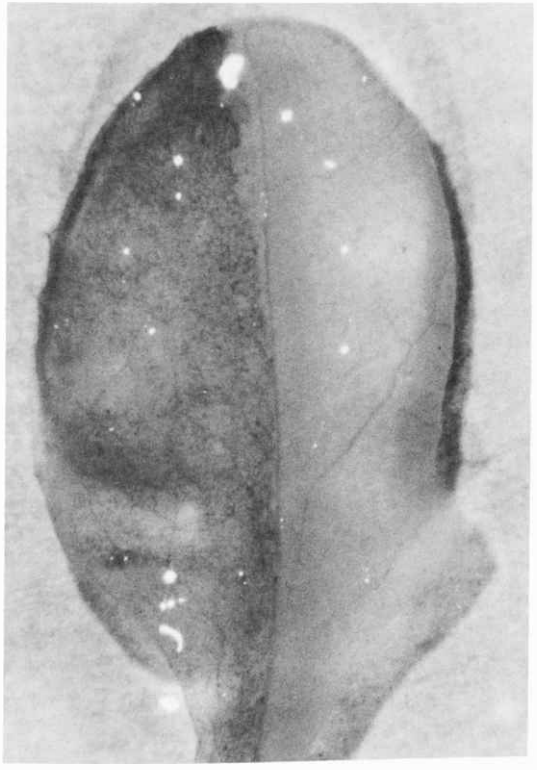

FIGURE 2 A leaf from an F1 plant heterozygous for gene-trap *DsG* and *Ac* transposase. The left half of the leaf comprises a sector in which a *DsG* element has transposed into a gene expressed in epidermal guard cells, which stain blue in X-Gluc.

Immunolocalization of the Hcf106 Membrane Protein in Maize Chloroplasts

A. Yonetani, A. Baron, R. Martienssen [in collaboration with R. Derby and D. Spector, Cold Spring Harbor Laboratory, and L. Dolan, John Innes Institute Norwich, England]

The *hcf106* mutant of maize is a nonphotosynthetic mutant that fails to accumulate chloroplast membrane protein complexes required for electron transport. Mutant chloroplast membranes have lost the lateral morphological heterogeneity associated with wild-type chloroplast membranes. We have cloned the *hcf106* gene using the Robertson's *mutator* transposable element as a tag, and last year, we reported the cDNA sequence of the Hcf106 gene product. It encodes a 27-kD precursor protein that migrates aberrantly as a 35-kD in vitro translation product on SDS-PAGE. We have raised polyclonal antibodies against a TrpE::Hcf106 fusion protein and purified them by affinity to a MalE::Hcf106 fusion protein, both overexpressed in *Escherichia coli*. The purified antibodies

detect a 30-kD mature protein that was localized to chloroplast membranes by subcellular fractionation and Western blotting. Although the protein sequence has no significant homology with known proteins, it has a putative membrane-spanning domain close to the amino terminus, and the protein is recovered quantitatively from chloroplast membranes that have been sonicated in high salt. This indicates that it is probably an integral membrane protein, as suggested by the sequence.

We have attempted to further localize the protein by immunoelectron microscopy. Preliminary attempts using the unpurified antibody gave strong labeling in the chloroplast thylakoid membranes, but they proved to be difficult to reproduce. Later experiments using the purified antibody have confirmed at least some of the earlier results. Maize is a C4 plant and has two main types of photosynthetic cells in mature leaves: the mesophyll and bundle-sheath cells. Both cell types have differentiated chloroplasts, but only the mesophyll chloroplasts have large thylakoid "stacks" that are clearly distinguished from the unstacked "stromal" lamellae. The Hcf106 protein is found in both cell types but is enriched in the stacked membranes of mesophyll cell chloroplasts. This location is consistent with the protein being required for the formation or the maintenance of these structures: Chloroplasts from mutant *hcf106* leaves lack thylakoid stacks and form large, uniform whorls of membranes instead. However, the presence of the protein in other chloroplast membrane fractions and in other (nonphotosynthetic) plastid types suggests that the function of the protein is not limited to these distinctive membrane domains. We hope to improve the resolution and sensitivity of these experiments in the coming year and complete a survey of other plastid membrane fractions.

Structure of the *Hcf106* Locus in Maize

A. Baron, R. Martienssen

We have determined the structure of the *Hcf106* locus by partial sequencing of a genomic clone (from a library provided by G. Johal and S. Briggs). There are five exons and four introns: The introns are approximately 0.2, 4.5, 0.5, and 0.4 kb in length, respectively, and have consensus plant intron donor and acceptor splice sites. The mature transcript is approximately 1.1 kb, so that the entire gene spans about 6.7 kb. This is the approximate size of a high-molecular-weight transcript detected by Northern analysis in some of our total RNA preparations. This transcript is not detected in RNA from mutant cells and is severely reduced in polyadenylated mRNA, suggesting that it is probably a precursor.

Structure and Function of the *Iojap* Gene in Maize

C.-D. Han, R. Martienssen [in collaboration with E.H. Coe, USDA-ARS, University of Missouri]

The *Iojap* gene in maize is required for the normal differentiation of photosynthetic chloroplasts in certain tissues of the maize plant. Homozygous mutant *iojap* plants have a variety of phenotypes characteristic of different genetic backgrounds. These phenotypes range from a subtle "grainy" phenotype to the loss of leaf margins, but typically, *iojap* plants have variegated leaves and transmit defective chloroplasts through a proportion of their female gametes. The pattern of variegation indicates that defective chloroplasts are found in groups of cells that arise around the time of leaf initiation and are preferentially located at the margins of the leaf. This has led to the suggestion that these cells are derived from the LI, or outer layer of the shoot apical meristem. Periclinal (or out-of-plane) divisions at the time of leaf initiation are known to lead to similar patterns of leaf variegation in other monocots in which it is possible to construct periclinal chimeras. However, such plants have not been unequivocally identified in maize.

Last year, we reported the cloning of the *Iojap* gene, using an allele generated by insertion of a *Mu1* transposable element. The *Iojap* gene encodes a 229-amino-acid open reading frame that has no significant homology with known genes. Genomic clones have been isolated from the maize inbred B73 (from a library provided by G. Johal and S. Briggs). The gene has four introns of approximately 0.1, 0.3, 0.1, and 9.1 kb, respectively. The reference allele, first described by Jenkins in 1924, was found to contain a *Ds*-like transposon inserted into the first exon. Excision of this 1.5-kb transposon leads to revertant somatic sectors and rare germinal revertant progeny. Excision is associated with restoration of wild-type transcript levels and restoration of the putative *Ij* reading frame. A second transcription unit lies ap-

proximately 2.0 kb upstream of the *Iojap* gene and encodes a 2.4-kb transcript transcribed in the same direction. It has no homology with *Iojap* and is transcribed in mutant, wild-type, and revertant tissue. This second transcript encodes a 624-amino-acid open reading frame that has no homology with known proteins.

Last summer, we performed a clonal analysis of *Iojap* gene function using two different marker genes required for anthocyanin pigmentation in the leaf. Anthocyanin pigmentation is restricted to the epidermal cells of the leaf blade but is also found in subepidermal cells in the leaf sheath, so that the origin of sectors that have lost anthocyanin pigmentation can be traced to one or the other cell layer (McDaniel and Poethig, *Planta 175:* 13 [1988]). Seeds heterozygous for translocations that link either the *Pl* gene (on chromosome 6L) or the *Bz* gene (on 9S) with the *Iojap* gene (on 7L) were irradiated with varying doses of γ-rays at Brookhaven National Laboratories last summer; 2000 kernels were planted, and mature plants were examined for *iojap* and anthocyanin-less sectors. Preliminary results suggest that *ij* sectors always include the epidermal layer, but these sectors were only found when the highest doses of γ-rays were used, so very few were obtained. Southern analysis of *ij* sectors of the uniform "grainy" phenotype, as well as analysis of revertant sectors in "grainy" leaves, suggests that the phenotype may not be fully cell-autonomous: Revertant sectors that do not penetrate the leaf genetically give fully green subepidermal layers, whereas radiation-induced sectors that do not include all cell layers are fully mutant. These results support the idea that *Ij* may be required in cells from only one layer but can influence the phenotype of their descendants, or their neighbors, in other layers. Clonal analysis of sectors in homozygous variegated plants, and in situ hybridization of revertant sectors, will provide a more stringent test of this hypothesis.

Isolation of Unstable Alleles of *ramosa1* in Maize

L. Goh (URP Program), A. Baron, R. Martienssen

The maize transposable element *Spm* (*Suppressor-mutator*) is a useful mutagen because of its ability to generate new derivative alleles at a high frequency. To obtain new alleles of *ij* using this transposon, approximately 55,000 F1 progeny from a cross between an *Spm* line and an *ij* tester were screened at the West Neck Road cornfield in the summers of 1991 and 1992. The *Spm* stock (provided by Bob Schmidt, University of California, San Diego) had a highly active *Spm* element at the *opaque2* locus on chromosome 7S. The tester stock was multiply marked on chromosome 7 with *o2*, *glossy1*, *ramosa1*, and *ij*. *ra*, *gl*, and *ij* are 16, 20, and 36 map units, respectively, from the *o2* locus and are on the long arm of chromosome 7. Only one *ij* plant was recovered out of 55,000 F1 plants, and analysis of linked markers indicated that this plant was an androgenetic haploid. However, three independent *ramosa* plants were recovered by screening mature plants for highly branched tassels (see below). Allelism tests confirmed that these plants had new alleles at the *ra1* locus.

The *RAMOSA1* gene is a meristem identity gene required for the determination of branch primordia as spikelet meristems in maize inflorescences. Maize flowers are formed via a succession of "switch-points," resulting in the differentiation of different types of meristems as they bud from one another: Floral meristems arise from spikelet meristems, which arise from spikelet pair meristems, which arise from branch primordia, which arise from the primary inflorescence meristems. This is a more complex situation than that found in plants such as *Antirrhinum* and *Arabidopsis*, where mutation in only one or two genes can lead to conversion of floral into inflorescence meristems. In maize, *ramosa1* ears and tassels have indeterminate branches at the location normally occupied by spikelet pairs. However, normal spikelets can develop from these ectopic branches, so that the plants are fertile.

All three new alleles of *ra1* were unstable and frequently reverted to the wild-type (unbranched) phenotype in ear and tassel sectors. An example of a mutable ear is shown in Figure 3. The origin of the mutability in each case has not yet been determined, but germinal reversion from one of these alleles has been shown to be very high (30–40% of *ra-m* gametes). Genetic tests for *Spm* activity at the locus are under way, but molecular analysis has failed so far to reveal any *Spm*-homologous sequence linked to the first mutable allele. Another mutable allele of *ra1* was obtained in a similar screen by Bob Schmidt (University of California, San Diego), who has kindly made it available. It differs from our alleles in that the tassel has a stable mutant phenotype, although revertant ears are frequently observed. It is not

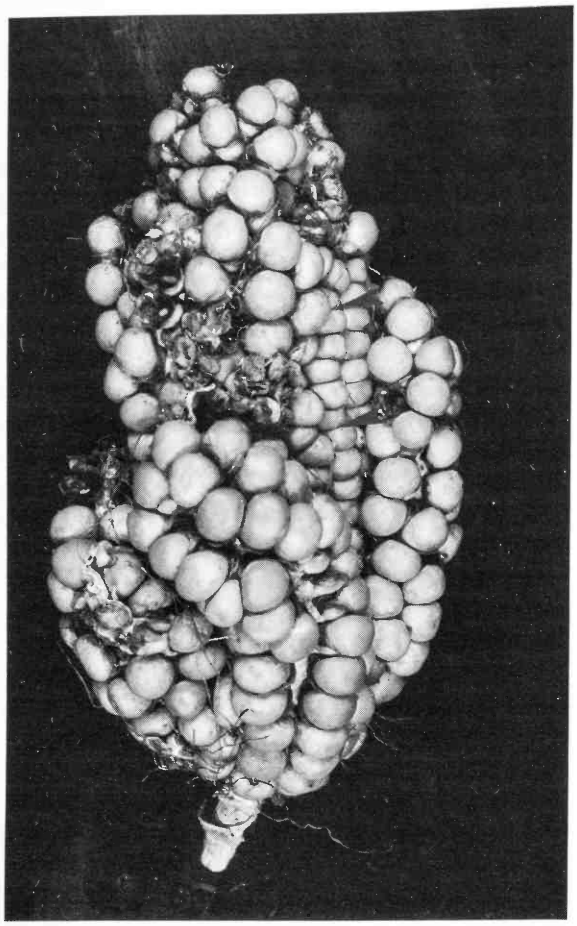

FIGURE 3 An ear from a plant heterozygous for *ramosa1* and a new unstable allele, *ra1-m*. The ear is highly branched, except for revertant unbranched sectors that have a normal arrangement of kernels (arrows).

caused by the insertion of an autonomous *Spm* (R. Schmidt, pers. comm.).

Establishment of Division Sites in Plant Development

J. Colasanti, V. Sundaresan [in collaboration with S.-O. Cho and S. Wick, University of Minnesota]

Since plant cells cannot migrate, it is necessary for plants to regulate both the timing and direction of cell division during plant development, i.e., a plant cell has to know both when to divide and which way to divide. The details of the process by which the plane of cell division is regulated are poorly understood at the molecular level. For example, it is known that the choice of the division site is made before mitosis and is precisely indicated by a band of microtubules called the preprophase band (PPB) and that this choice is imprinted in the cellular memory during cytokinesis since the PPB disappears before metaphase. However, the mechanism of this imprinting is unknown, although it has been proposed that this is accomplished by F-actin, which is associated with the PPB in some types of plant cells. Last year, we reported that the $p34^{cdc2}$ kinase homolog of higher plants colocalized with the PPB band of microtubules that predicts the future division site in premitotic cells of the maize root tip. This observation suggested to us that the determination of the plane of cell division may be mediated by the $p34^{cdc2}$ kinase. We have now extended on our previous observations. We find that when the microtubule PPB is disrupted using the drug oryzalin, $p34^{cdc2}$ is no

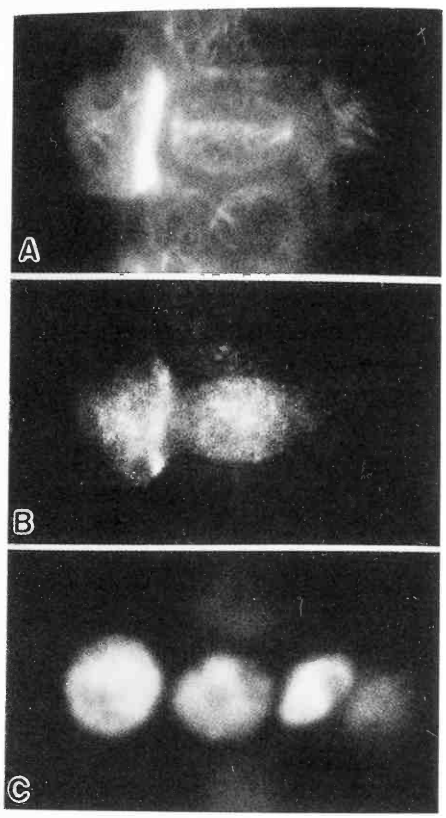

FIGURE 4 Immunofluorescence staining of leaf epidermis showing a developing stomatal complex. Cells are stained with tubulin antibody, which reveals the transverse interphase band in the guard mother cell (GMC) and the more brightly stained preprophase band in the subsidiary mother cell (SMC) on the left (*A*), maize $p34^{cdc2}$ antibody (*B*), and DAPI (*C*). Magnification, 1600x.

longer associated with the division sites, although it is able to leave the nucleus. Therefore, it is likely that the function of the PPB is to direct $p34^{cdc2}$ to the division site. Second, we have examined progenitor cells of the stomatal complex of the leaf epidermis. In the grasses, the formation of this complex involves some highly asymmetric cell divisions, and furthermore, F-actin is not associated with the PPB in these cells (Cho and Wick, *J. Cell Sci. 92:* 581 [1989]). We have now shown that the association of $p34^{cdc2}$ with the PPB also occurs in the stomatal complex progenitor cells, both in maize and in rye (Fig. 4). As in the case of the root tip cells, the association of $p34^{cdc2}$ with the PPB seems to occur late in G_2/M, which is consistent with the idea that the PPB forms first and then directs $p34^{cdc2}$ to the division site. Our current model is that the division site is modified by $p34^{cdc2}$ by phosphorylation of cortical substrates. In this model, the phosphorylation of cortical substrates at the point of contact of the PPB is the mechanism by which the division site is "imprinted" in the cellular memory, and which is used to direct the expanding cell plate to the division site at telophase.

Cyclins in Higher Plants

J.-P. Renaudin, J. Colasanti, Z. Yuan, V. Sundaresan
[in collaboration with C. Jessus and H. Rime, CNRS, France]

The subcellular distribution of $p34^{cdc2}$ is believed to be regulated by the associated cyclin, and it is likely that there may be a specific cyclin required for association of $p34^{cdc2}$ with the division site. For this reason, we have initiated a study of plant mitotic cyclins, and we have now isolated clones for seven cyclins from maize (from a cDNA library constructed by B. Veit and S. Hake, USDA, Albany), which fall into four distinct classes numbered type 1 through type 4, in order of decreasing abundance. We have isolated full-length or near-full-length clones for one member of each class and have demonstrated their functionality by their ability to induce maturation in oocytes, by microinjection of sense RNA from each clone. Sequence analysis of the clones shows that they all have homologies with both A and B animal mitotic cyclins but cannot be classified as clearly A-type or B-type. However, types 1, 3, and 4 can be termed "B-like" because they are slightly more homologous to animal cyclin B, and type 2 can be termed "A-like" because it is slightly more homologous to animal cyclin A. In the meantime, the sequences of three other plant cyclins have been published, one each from soybean, carrot, and *Arabidopsis*. Our data show that these cyclins are prototypes of different classes of cyclins that are found within a single plant species. The type-1 and -3 cyclins are homologous to the soybean and *Arabidopsis* cyclins, the type-2 cyclin is homologous to the carrot cyclin, and the type-4 cyclin is in a class by itself. All four cyclins that we have cloned from maize are preferentially expressed in proliferating tissues.

The maize cyclins have been mapped to the maize restriction-fragment-length polymorphism (RFLP) map by Keith Rufener (ICI Seeds, Iowa). Interestingly, the type-4 cyclin clone maps close to a locus called *polymitotic*, which was first identified by George Beadle in 1930 as a meiotic mutant in which supernumerary cell divisions occur after the first meiotic division. We are now investigating whether the *polymitotic* mutation may be a mutation in the type-4 cyclin. We are also generating antibodies to the different cyclins to study their localization within dividing cells.

Analysis of Plant γ Tubulins

J. Colasanti, V. Sundaresan

The α and β tubulins comprise the structural subunits of microtubules. The placement of microtubules within cells determines cellular architecture, and their dynamic movements are essential for many mitotic functions. Recent studies in animal and fungal cells have shown that a third class of tubulin, the γ tubulins, has a fundamental role in cytoskeletal organization. Specifically, γ-tubulin protein is localized to centrosomes and spindle pole bodies in animal cells and fungal cells, respectively. This suggests that γ tubulin is involved in microtubule nucleation—an essential function for correct chromosome separation and mitosis in general. In plants, cytoskeletal structure is also important for the determination of division polarity and morphogenesis. Although the cells of higher plants lack a defined spindle pole or centrosomal region found in animal and yeast cells,

they do possess several unique microtubule-containing structures. The preprophase band (PPB) is a dense band of microtubules that forms in the cortex of the cell before the onset of mitosis and disappears before the mitiotic spindle is formed. The position of the PPB within the cell is somehow "imprinted" in cellular memory, as it predicts where the future wall will form to divide the daughter cells. It has been suggested that at least in some plant cells, the PPB is set up by microtubule nucleation at the cell cortex. Furthermore, at telophase, the new cell plate is generated by a different microtubule structure called the phragmoplast, which has been shown to be associated with microtubule nucleation. Since γ tubulin is found at centers of microtubule nucleation, in order to identify the centers of plant microtubule nucleation during mitosis, we have initiated experiments to study the localization of γ tubulin in plant cells. We have taken advantage of the conserved nature of γ tubulin between diverse species to isolate the maize homolog. A polymerase chain reaction (PCR) fragment generated by degenerate primers was used as a probe to isolate seven partial clones from a maize cDNA library derived from immature ears (a gift from B. Veit and S. Hake, USDA). Sequence analysis of these cDNAs shows that they fall into two distinct types. Although they are nearly 90% identical at the amino acid level, one of the clones has a 27-amino-acid deletion near the middle of the deduced protein. Southern blot analysis indicates that maize may have up to four γ tubulin genes. Preliminary Northern blot analysis indicates that the 1.6-kb γ-tubulin transcript is found at higher levels in apical meristem, root tip, and young leaf tissue than in mature leaf tissue, indicating that there is a correlation between the proliferative capacity of the tissue and mRNA levels. At the present time, we are producing polyclonal antibodies against a portion of the γ-tubulin protein, with the aim of determining the localization of γ tubulin within plant cells by immunofluorescence microscopy.

Is *Mu* Element Transposition Replicative or Conservative?

V. Sundaresan, R. Srinivasan

The *Mu* elements of maize show high rates of forward transposition but low rates of germinal excision, suggesting that they may transpose by a replicative mechanism. If so, this would make the *Mu* transposon system unusual, since all of the other eukaryotic transposons studied in detail (except the retrotransposons) appear to transpose by a conservative or "cut-and-paste" mechanism. In some transposon systems (the *P* elements of *Drosophila*, and the Tc1 elements of *Caenorhabditis elegans*), the excision of the elements is followed by double-strand gap repair using the element on the homologous chromosome, so that they appear to generate forward transpositions without excisions. However, when the wild-type sequence is present on the homologous chromosome, the reversion rate is increased by more than 100-fold, due to gap repair using the wild-type sequence as a template. We investigated whether a similar mechanism was operating in *Mu* element transposition, i.e., whether *Mu* element excision was being masked by double-strand gap repair. If so, we might expect to see significantly increased rates of reversion if a gene containing a *Mu* insertion is heterozygous with the wild-type gene. We used a line that contains a *Mu1* element in the *Bronze1* (*Bz1*) gene. This allele (called *bzMum9*) was linked to *Shrunken* (*Sh*) and *waxy* (*wx*) and was made heterozygous over the wild-type gene *Bz1* linked to *Sh* and *wx*. We then examined the frequency of kernels that were purple and carried the *Sh* and *wx* markers in crosses to recessive testers. Since double crossovers are suppressed by chiasma interference in this interval, such kernels should arise at low rates unless there is a high rate of gene conversion using the wild-type *Bz1* gene as template following *Mu* excision. The cross was *Sh bzMum9 wx/sh Bz Wx* x *sh bz wx/sh bz wx*.

More than 20,000 kernels from this cross were examined, and purple kernels that were *Sh wx* arose at a frequency of about 10^{-3}, which was approximately the same as the rate of double crossovers in this region (in control crosses). The germinal reversion rate of *bzMum9* is less than 10^{-4}, but the frequency of forward transposition in this line is very high (>0.1 new insertions per *Mu1* element per generation), so that if the forward transposition was due to excision followed by gap repair using the chromosomal homolog, we may expect to see a reversion rate of about 10^{-1}. Thus, we conclude that double-strand gap repair using the homologous chromosome does not occur at sufficiently high rates to account for the discrepancy between forward transposition and reversion rates of *Mu* elements. Instead, our data support a replicative model for *Mu* element transposition.

Differential Transcriptional Regulation by the Maize *P* Gene

E. Grotewold, T. Peterson [in collaboration with B. Bowen and B. Drummond, Pioneer Hi-Bred International, Inc., Johnston, Iowa]

Certain viruses that cause leukemia carry an oncogene termed v-*myb*, which is responsible for the neoplastic transformation of myelomonocytic hematopoietic cells. The v-*myb* gene is a truncated form of the cellular c-*myb* gene, which is required for the normal proliferation and development of hematopoietic cells. Both v-*myb* and c-*myb* encode sequence-specific DNA-binding proteins that activate transcription. *myb*-homologous genes have been identified in a variety of organisms, but their functions in most cases are unknown. We recently discovered that the maize *P* gene encodes a *Myb*-homologous protein (Grotewold et al., *Proc. Natl. Acad. Sci. 88:* 4587 [1991]). Because the *P* gene is known to regulate the synthesis of flavonoid pigments, we can now examine the function of a *Myb*-homologous protein in a well-characterized biosynthetic pathway that is amenable to genetic analysis.

The *P* gene regulates the expression of three genes (*C2*, *CHI*, and *A1*) that encode enzymes for phlobaphene pigment biosynthesis. These three structural genes are also required for the formation of anthocyanin pigments but are not sufficient, and other genes are required including *A2*, *Bz1*, and *Bz2*. These latter genes are not regulated by *P*, but by the maize *Myb* homologs *C1* or *Pl* in conjunction with either *R* or *B*, which encode basic helix-loop-helix proteins. Our aim was to determine the mechanism by which *P* regulates a subset of the structural genes for flavonoid biosynthesis, and for this purpose, we compared the interaction of *P* with *A1* (regulated by *P*) and *Bz1* (not regulated by *P*).

The *P* gene produces at least two alternatively spliced transcripts (Grotewold et al., *Proc. Natl. Acad. Sci. 88:* 4587 [1991]). We tested each *P* transcript for its ability to activate transcription by using microprojectile bombardment to introduce *P* cDNA clones together with the *A1* or *Bz1* target promoters linked to a luciferase reporter gene into maize cells in transient transformation assays. The largest cloned *P* cDNA (*P*-cDNA1) efficiently activates the *A1* promoter but not the *Bz1* promoter. We also found that *P* does not require *R* for the *trans*-activation of *A1*, nor does *R* alleviate the inability of *P* to activate *Bz1* efficiently. In contrast, *C1* requires *R* for efficient *trans*-activation of both *A1* and *Bz1*. Thus, although *P* and *C1* encode proteins with very similar *Myb*-homologous DNA-binding domains, and they regulate an overlapping set of target genes, *P* and *C1* may activate transcription through very different mechanisms. The smaller *P* cDNA (*P*-cDNA2) has no apparent activating or inhibiting function; this is not surprising, since *P*-cDNA2 encodes a protein with a truncated *Myb*-homologous domain (Grotewold et al., *Proc. Natl. Acad. Sci. 88:* 4587 [1991]) which would not be expected to bind DNA.

The *P* gene could activate transcription of the flavonoid biosynthetic genes directly (by binding to the target gene promoters) or indirectly (by first activating an intermediate factor which then activates the flavonoid biosynthetic genes). To test these different models, we examined the DNA-binding activity of the protein encoded by *P*-cDNA1. *P*-cDNA1-coding sequences were expressed in *Escherichia coli* and used for gel-retardation experiments using as a probe the 220-base-pair fragment of the *A1* promoter previously shown to confer *P* inducibility. The results show specific binding of *P*-encoded proteins to the *A1* promoter fragment in vitro, in the absence of any other maize protein. This is the first demonstration of a direct interaction between a plant *Myb*-homologous protein and a target gene promoter and thus supports the idea that *P* directly activates the genes for flavonoid biosynthesis.

We determined the binding sites of the *P*-encoded protein in the *A1* promoter by biochemical analyses of protein-DNA complexes, and we are now testing the role of these binding sites in *A1* regulation by *P*. The binding sites of *P* in *A1* are considerably different from the sequences to which vertebrate *Myb* protein binds, as well as from the *Bz1* promoter sequences required for regulation by *C1* and *R*. We further characterized *P*- and *Myb*-binding sites through parallel binding selection experiments, and the results show a clear difference in specificity between *P*- and *Myb*-encoded proteins: *P* binds preferentially the sequence $CC^{T}/_{A}ACC$, and this same sequence motif is present twice at the *P* binding site in the *A1* promoter. In contrast, *Myb* binds the sequence $^{C}/_{T}AACGG$. These results show for the first time that *Myb*-homologous proteins with similar DNA-binding domains can have very different binding specificities.

Why does *P*, unlike its homolog *C1*, not activate *Bz1* in vivo? Gel-retardation assays showed that the *P*-encoded protein binds very poorly, if at all, to a 200-bp fragment of the *Bz1* promoter that is required

for regulation by *C1* and *R*. This finding is sufficient to explain the lack of activation of the *Bz1* gene by *P*. To test this hypothesis in vivo, we constructed a chimeric protein that carries the *Myb*-homologous DNA-binding domain of *P* fused to the transcriptional activation domain of *C1*. This *P-C1* fusion activates *A1* even in the absence of *R* but does not efficiently activate *Bz1*. Thus, no additional protein factors interacting with the *P*-encoded protein need be postulated to explain the differential regulation of the *A1* and *Bz1* promoters. However, it remains possible that *P*, like its homolog *C1*, interacts with additional specific factor(s) to activate the target genes *A1*, *C2*, and *CHI*. Experiments to identify and characterize such factor(s) are in progress.

ORGAN-SPECIFIC GENE EXPRESSION IN MAIZE: THE *P-wr* ALLELE

We have recently shown that the maize *P* gene encodes a *Myb*-homologous regulator of genes for flavonoid pigment biosynthesis (Grotewold et al., *Proc. Natl. Acad. Sci. 88:* 4587 [1991]). The *P*-regulated pigments are localized to specific floral organs, including the pericarp (outer layer of the kernel) and cob glumes (floral bracts subtending the kernel). Some alleles of *P* elicit differential pigmentation of the pericarp and cob glumes, and these are identified by a suffix indicating their pattern of pigmentation in these floral organs (Table 1).

We are interested in determining the mechanism(s) of organ-specific expression of the *P-wr* allele. Northern blot analysis indicates that *P-wr* transcripts are present not only in the pigmented cob glumes, but also in the colorless pericarps. However, *P-wr* transcripts are not detectable in nonpigmented vegetative organs such as leaves. Thus, the lack of pigmentation in pericarp, despite the presence of *P-wr* transcripts, is specific to that tissue.

To test whether the pericarp and cob transcripts of *P-wr* can activate transcription of the structural genes for flavonoid pigment biosynthesis, Northern blots of pericarp and cob glume RNAs were hybridized with probes from the *C2* and *A1* genes, which encode enzymes for the first and third committed steps in flavonoid biosynthesis. The *A1* probe detected transcripts in both pericarp and cob glumes at 10 DAP (days after pollination); at 21 DAP, the transcripts were present in cob glumes but not pericarp. In contrast, the *C2* gene transcripts were present in both pericarp and cob glumes at both 10 and 21 DAP. The *C2* transcript level appeared lower in the pericarp of *P-wr* when compared to *P-rr* but higher in the cob glumes of *P-wr* compared to *P-rr*. These preliminary results suggest that the failure to produce pigment in the pericarp of *P-wr* plants is due to the inability of the *P-wr*-encoded transcripts to *trans*-activate the *A1* gene (and possibly other genes required for pigment synthesis). Our current aim is to isolate *P-wr* cDNAs from pericarp and cob glumes and compare them to see whether different transcripts are produced by the *P-wr* allele in each organ. We will then use microprojectile bombardment to express transiently each cDNA in pericarp and cob glumes; the results should indicate whether organ specificity is encoded within each cDNA or whether it is due to differential localization of factors that interact with the *P-wr*-encoded protein(s).

TABLE 1 Differential Pigmentation of the Pericarp and Cob Glumes of *P* Alleles

Allele	Pericarp	Cob glumes
P-rr	Red	Red
P-rw	Red	White (colorless)
P-wr	White (colorless)	Red
P-ww	White (colorless)	White (colorless)

Ac Transposition Mechanism Inferred from Twinned Pericarp Sectors

P. Athma, T. Peterson

The transposable element *Ac* is a versatile tool for mutagenizing and cloning genes in maize and other plants. We used *Ac* insertional mutagenesis to define the limits of the *P* gene genetically (Athma et al. 1992), and we are currently using *Ac* to generate mutations at specific sites within the *P* gene. We are also analyzing a number of new *P* alleles resulting from short-range *Ac* transpositions to learn more about the mechanism of *Ac* transposition. The kernel pigmentation phenotype conditioned by the maize *P* gene is an excellent visual indicator of the effects of the *Ac* element on gene expression. The *P* gene system has the additional advantage that one can easily detect adjacent patches of phenotypically distinct pericarp known as twinned sectors. Such twinned

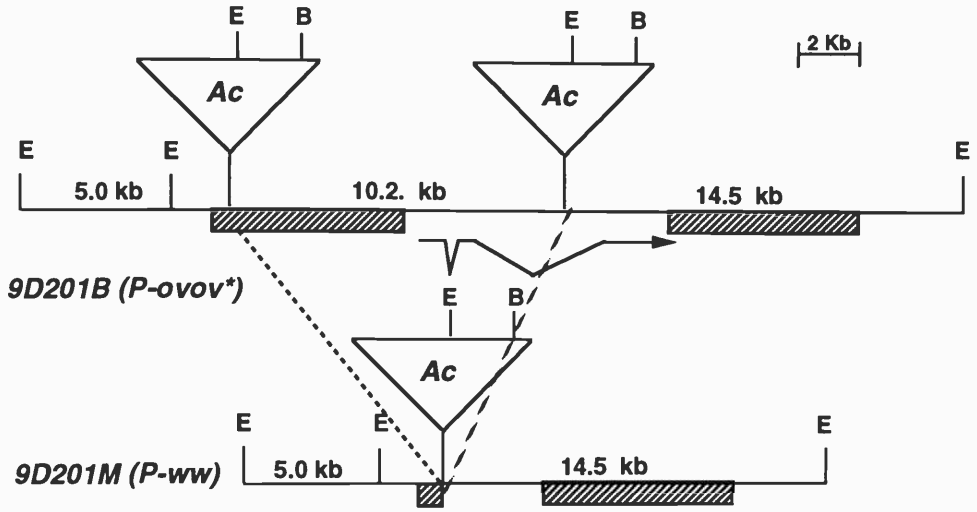

FIGURE 5 Structures of the *P*-9D201B* and *P*-9D201M* alleles derived from a twinned pericarp sector. The dashed lines indicate the 10.2-kb deletion in *P*-9D201M*.

sectors are formed when a genetic event at the *P* locus (such as *Ac* transposition) generates two nonidentical daughter chromosomes, which are segregated into two adjacent daughter cells. When the progeny of these cells are included as paired clones within the pericarp, a twinned sector develops. Twinned sectors can encompass one or many kernels, depending on the time during development when the progenitor twinned cells arise. The alleles that produce the twinned sector can be recovered from the progeny of the kernels within each twin. Twinned sectors are especially valuable because one can analyze both chromosomal products arising from a single genetic event and reconstruct the molecular events leading to twin formation.

One particularly informative twinned sector was derived from the *P-ovov* allele, which confers orange-variegated pericarp and cob glumes. The multikernel sector had white pericarp twinned with a sector of pale-orange variegated pericarp. Molecular analysis indicated that the allele specifying light-orange variegated pericarp (*P*-9D201B*) had two *Ac* elements in the *P* locus: one *Ac* at the original site in the progenitor *P-ovov* allele and a second *Ac* element at a site 10.2 kb upstream. The allele from the twinned white sector (*P*-9D201M*) carried a single *Ac* inserted at the breakpoint of a 10.2-kb deletion of the *P* locus (Fig. 5). Sequence analysis showed that the deletion endpoints were precisely at the sites of *Ac* insertion (Fig. 6). It is formally possible that the

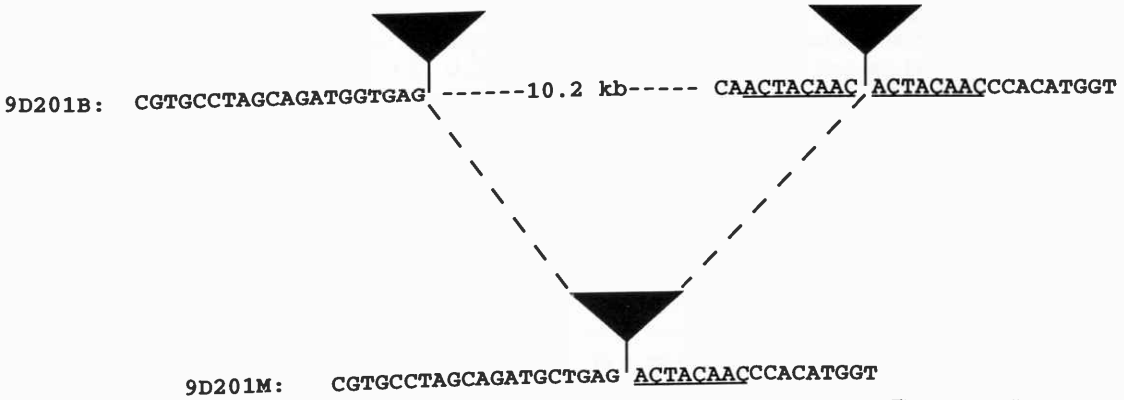

FIGURE 6 DNA sequences at the sites of *Ac* insertion in *P*-9D201B* and *P*-9D201M* alleles. The dashed lines indicate the deletion in *P*-9D201M*. The 8-bp target site duplication is underlined.

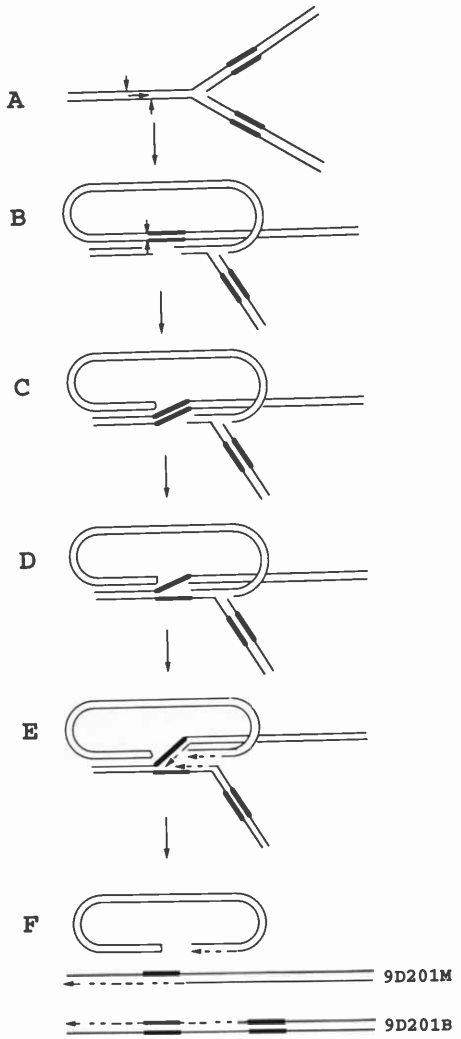

FIGURE 7 (*A*) The *P* locus during chromosome replication. Solid bars indicate the *Ac* element in the *P* locus. The *Ac* transposase makes a double-strand staggered cut at the target site located ahead of the replication fork. (*B*) Chromatid with the donor *Ac* element associates with the target site, and the transposase makes a double-strand cut at one end of the *Ac* element. (*C*) The cut end of *Ac* is ligated to one free end of the target site. At this point, the *Ac* element bridges the target site and the donor site. (*D*) In a normal transposition, both DNA strands of the opposite end of *Ac* would be cut and ligated into the target site, and then the ends of the donor site would be ligated together. To generate the *P*-9D201B/M* twinned sector, only one strand of *Ac* is ligated to the target site, and the other strand remains attached to the donor site. (*E,F*) Dashed lines with arrows indicate DNA replication. Replication through the lower strand generates the *P*-9D201B* daughter chromosome with two copies of *Ac*. Replication of the upper strand releases the acentric fragment derived from the 10.2 kb of DNA between the *Ac* donor and target sites (fragment in *F*). Template switching to the free end formerly joined to *Ac* and continued replication generate the deletion allele *P*9D201M*.

P-9D201M* allele could have been derived from the *P*-9D201B* allele by recombination between the two *Ac* elements that are in direct orientation. However, to generate the observed twinned sector, such a recombination would have had to occur immediately after the initial transposition that generated the *P*-9D201B* allele. In fact, progeny testing of the *P*-9D201B* allele indicates that such recombinations are rare, if they occur at all. Therefore, we propose that the twinned sector resulted from an aberrant transposition event as shown in Figure 7 (a similar model was proposed for transposition of the *Antirhinnum Tam3* element; Robbins et al., *EMBO J. 8:* 5 [1989]).

The salient features of this *Ac* transposition model are that (1) *Ac* transposes during DNA replication, (2) there is a physical association of the *Ac* donor and target site loci, and (3) the *Ac* termini are transferred sequentially from the donor to the target site. This transposition model fits genetic and molecular data showing that *Ac* often transposes during DNA replication from a replicated donor site to an unreplicated target site. Additionally, the association of donor and target site loci proposed here can help to explain the observation that *Ac* tends to transpose to nearby sites. Analysis of 11 other twinned sectors derived from *P-ovov* have provided further insight into the mechanism of *Ac* transposition (P. Athma and T. Peterson, in prep.).

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ARABIDOPSIS SIGNAL TRANSDUCTION AND FLOWER DEVELOPMENT

H. Ma C. Flanagan H. Huang R. Ram
Y. Hu R. Lavi M. Tudor
C. Huang Y. Mizukami C. Weiss

Our research continues to emphasize two main areas: characterization of G-protein function in plant signal transduction pathways and analysis of a family of genes involved in flower development and other functions. Plant cells respond to a variety of external and internal signals, but little is known about the molecular mechanisms of the plant signal transduction pathways involved. To begin to understand plant signaling processes, we have taken the approach of studying G proteins, which, in animals and simple eukaryotes, are known to play important roles in signal transduction. We have previously isolated an *Arabidopsis* gene (*GPA1*) encoding a G-protein α subunit (Ma et al., *Proc. Natl. Acad. Sci. 87:* 3821 [1990]), and we are continuing our characterization of *GPA1*. We have recently obtained detailed information on its expression pattern, providing insights into the possible function of *GPA1*.

Flower morphogenesis is a complex developmental process. In recent years, a number of *Arabidopsis* floral homeotic genes have been characterized (Coen and Meyerowitz, *Nature 353:* 31 [1991]). Mutations in one of these genes, *AGAMOUS* (*AG*), cause double-flower phenotypes, where the stamens are converted to extra petals and the ovary is replaced by a new flower. The *AG* DNA sequence (Yanofsky et al., *Nature 346:* 35 [1990]) indicates that it encodes a protein with strong similarity to the DNA-binding domains of transcription factors from humans (SRF) and yeast (MCM1), suggesting that the AG protein is a transcription factor (Fig. 1). This domain is also found in another floral homeotic gene, *DEF A* from *Antirrhinum majus* (Sommer et al., *EMBO J. 9:* 605 [1990]), and has been referred to as the MADS-box (Fig. 1) for *M*CM1-*A*G-*D*EFA-*S*RF (Schwarz-Sommer et al., *Science 250:* 931 [1990]). Additional MADS-box genes were isolated using *AG* as a probe, and designated *AGL1* through *AGL6* for *AG-L*ike (Fig. 1; Ma et al., *Genes Dev. 5:* 484 [1991]). We have been focusing our efforts on *AG*, *AGL1*, *AGL2*, and *AGL3*.

The approaches we have taken to study the MADS-box genes and *GPA1* are summarized in the 1990 and 1991 Annual Reports. Two postdoctoral fellows, H. Huang and C. Weiss, are the major forces in the work on *GPA1*. They and two other postdoctoral fellows, C. Flanagan and Y. Mizukami, as well as a technician, Y. Hu, are involved in various studies on the MADS-box genes. In addition, C. Huang, a graduate student in the Genetics Program, did a rotation in our laboratory, working with H. Huang on AG in vivo DNA-binding studies; R. Lavi was a participant in the "Partners for the Future" program for talented high school students, and he worked with C. Weiss; R. Ram was an URP, and she worked on analysis of wild-type and *ag* floral RNAs; M. Tudor was an undergraduate student who performed in vitro experiments with AG and AGL1 proteins. Here, we describe our recent progress during the past year in the area of *GPA1* and MADS-box genes, as well as in a collaborative effort to establish an enhancer/gene trap transposon system in *Arabidopsis thaliana*.

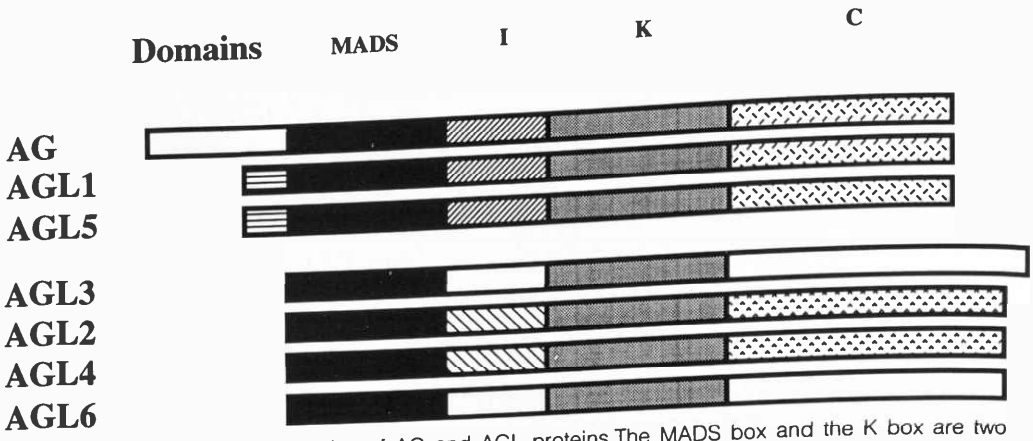

FIGURE 1 Schematic representation of AG and AGL proteins.The MADS box and the K box are two regions conserved in all of these proteins, whereas the interbox (I) and the carboxy-terminal (C) regions are only conserved between more similar members, as represented by same type of shaded bars. The open bars are not conserved. (Modified from Fig. 5A of Ma et al., *Genes Dev. 5:* 484 [1991].)

Analysis of *GPA1* Expression Using a Translational Fusion to a Reporter Gene

H. Huang, H. Ma

Because the developmental stage and localization of expression may suggest what the function of a particular gene is, we are interested in characterizing the temporal and spatial expression pattern of the *GPA1* gene. The expression of many G proteins is known to be regulated; for example, rod and cone transducins are expressed specifically in rod and cone photoreceptors, respectively, and the olfactory G protein is expressed only in the olfactory epithelium. Previous experiments indicated that *GPA1* mRNA is found in both vegetative and floral organs of *Arabidopsis*. To obtain more detailed information about *GPA1* expression, we have constructed a translational fusion between the *GPA1* genomic DNA and the bacterial *uidA* gene (encoding glucuronidase, or GUS) and introduced it into *Arabidopsis* plants via *Agrobacterium*-mediated transformation. We have carried out histochemical analysis of the *GPA1-uidA* transgenic plants (Huang et al., submitted [1993b]). Our results show that *GPA1* is expressed at root tips and cotyledons as early as 32 hours after seed germination. Later in developing seedlings, *GPA1* is expressed strongly in the shoot apex and in leaf primordium, as well as in root tips and new roots. In fully expanded cotyledons, rosette leaves, and cauline leaves, *GPA1* is more highly expressed in the vascular system but can also be detected in other cells. In floral stems of the adult plant, *GPA1* expression is localized mainly in the phloem and xylem of the vasculature.

In floral buds and mature flowers, carpels exhibit very strong GUS activity, and sepals and stamens have weaker but detectable *GPA1* expression. Finally, *GPA1* is also expressed in developing seeds, but the expression diminishes gradually to undetectable levels as the seeds mature. The fact that *GPA1* is expressed in several tissues and at various stages suggests that it may be involved in multiple signaling processes.

Analysis of the Distribution of the *GPA1* Gene Product

C. Weiss, H. Ma

We have also characterized the distribution of the *GPA1* gene product (GPα1) more directly using anti-GPα1 antibodies (Weiss et al. 1993). Results obtained so far show that the GPα1 protein is expressed in all tissues examined with the exception of mature seeds. It is expressed in roots, cotyledons, rosette leaves, cauline leaves, floral stems, flowers, seedpods, and during all stages of plant development. Interestingly, we have found that the level of GPα1 is higher in immature organs (i.e., growing leaves) compared to mature organs (full-size leaves). Im-

munolocalization results indicate that the GPα1 protein is localized predominantly in the vascular tissues of mature organs, especially in the cambium and phloem. In developing organs, GPα1 is present at a higher level in the root, leaf, and floral meristems, as well as in the leaf and floral organ primordia. During flower development, dividing microspores, but not mature pollen, show a high level of GPα1 protein. GPα1 is also present in nectaries, in developing ovules, and, during fertilization, in the growing pollen tube. After fertilization, it is present in developing embryos as early as the globular stage and in the seed coat. The results from these studies using antibodies agree very well with the information obtained using the reporter gene fusion (see above). Furthermore, the antibody studies provide detailed information about the GPα1 distribution during flower development and early embryo development. The association of the *GPA1* gene product with highly dividing tissues suggests that GPα1 in plants may be involved in cell division regulation, but further efforts are necessary to understand G-protein-mediated signal transduction in plants.

Analysis of *AGL3* Function

C. Weiss, H. Ma

Six members of the *Arabidopsis* MADS-box gene family were previously identified and designated *AGL1* through *AGL6*. Among these genes, *AGL3* is unique in that it is not flower-specific; it is also expressed in vegetative tissues. We have recently isolated and characterized the complete genomic and cDNA sequences of *AGL3* (Huang et al., submitted [1993a]). To understand the role of *AGL3* during plant development, we analyzed *AGL3* expression using Northern and in situ hybridizations, and the results show that *AGL3* is expressed in rosette leaves, floral stems, cauline leaves, and seedpods and at lower levels in flowers, but it is not expressed in roots. The fact that *AGL3* is expressed in a number of tissues suggests that it has a more general function than its floral counterparts. To study *AGL3* function, transcriptional fusions between a constitutive promoter and the *AGL3*-coding region, inserted in either sense or antisense orientation, were constructed and introduced into *Arabidopsis*. Preliminary results indicate that overexpression of the *AGL3* gene in the sense orientation creates a novel phenotype. Compared to wild-type plants, the transgenic plants show larger and more numerous flowers, as well as short, abnormal seedpods that contain fewer seeds than wild-type plants, indicating partial sterility. The transgenic inflorescence is also flatter than the wild-type one, suggesting that cell elongation or division might be affected in the pedicel of these flowers. On the other hand, transgenic plants carrying an antisense construct have no morphological defects, but flower about 2–3 weeks later than wild-type plants. These results suggest that the *AGL3* gene product might regulate a great number of target genes, which may be involved in very different processes of plant development.

Probing *AG* and *AP2* Interaction Using Transgenic *Arabidopsis* Plants

Y. Mizukami, H. Ma

In *ag* mutant flowers, reproductive organs (stamens and carpels) are converted to sterile perianth organs (petals and sepals), and there is an increase in the number of floral organs. In contrast, mutations in another floral homeotic gene, *AP2*, cause the replacement of the perianth organs by the reproductive organs and the reduction of floral organ number. Therefore, it has been proposed that AG negatively regulates *AP2* function. We have tested whether AG inhibits *AP2* function by ectopically expressing *AG* under the cauliflower mosaic virus (CaMV) 35S promoter in transgenic *Arabidopsis* plants. We have constructed a fusion between the 35S promoter and the *AG* cDNA and introduced the fusion into wild-type *Arabidopsis* plants. Our results (Mizukami and Ma 1992) show that many of the transgenic plants produce *ap2*-like flowers, which have carpels or carpelloid organs in the first whorl. In addition, petals and stamens are reduced in size and number or are absent in some transgenic flowers, similar to the *ap2* flowers. These results support the hypothesis that AG negatively influences *AP2* function. Using a homolog of *AG* from *Brassica* (*BAG1*) to transform wild-type tobacco plants, Yanofsky and colleagues obtained similar results where ectopic expression of *BAG1* leads to *ap2*-like flowers in the transgenic plants (Mandel et al. 1992).

Dissecting *AG* Functions Using an Antisense Construct

Y. Mizukami, H. Ma

Genetic evidence indicates that *AG* is involved in several aspects of floral morphogenesis: (1) negative interaction with *AP2*, (2) organ identity in the inner two whorls, and (3) determinacy at the center of the flower. A few mutant alleles of *AG* have been reported, and all of them have the same phenotypes. To begin to dissect these roles, we sought to disrupt *AG* function by using antisense RNA, which may produce different levels of inhibition due to different levels of antisense RNA. We constructed an *AG* antisense fusion to the 35S promoter and introduced it into wild-type plants. We have observed that transgenic plants with the *AG* antisense construct show a wide range of phenotypes (Mizukami and Ma, in prep. [1993]). Some have nearly normal flowers, with sepals, petals, stamens, and carpels. However, after more careful inspection, we have found that the ovary is enlarged compared to wild type, and hand dissection revealed another flower, as well as seeds, within the ovary. Furthermore, the inner flower has parts of a third flower within its ovary. Therefore, in these transformants, the level of AG activity allows the morphogenesis of stamens and carpels to produce fertile flowers, but it does not maintain the determinant growth pattern of a normal flower. It is possible that different aspects of *AG* function require different amounts of AG activity.

Functional Analysis of the AG Domains in Transgenic *Arabidopsis*

Y. Mizukami, H. Ma

Comparison of AG and other plant MADS-box gene products indicates that the AG protein can be divided into several regions, which may correspond to functional domains. *AG* function may vary due to differential interaction with protein factors present at different stages and/or in different cells. The interactions may or may not be mediated by different domains in the AG protein. We have begun to dissect the domains required for these functions by expressing truncated AG protein in transgenic plants and characterizing their effects in transgenic plants. Our results from transgenic plants with a construct expressing a truncated AG protein lacking the most carboxy-terminal domain show that the flowers from most transgenic plants have an indeterminant phenotype (flower within a flower); a smaller subset of these transgenic plants have abnormal carpel development, and still fewer transformants have conversion of stamens to petals, as in *ag* mutant flowers. The range of floral phenotypes is similar to that found in the transformants carrying the *AG* antisense construct. Because a large majority of the transformants have the *ag*-like phenotypes, cosuppression is not a likely explanation. Rather, we believe that the truncated AG protein somehow interferes with or inhibits the function of the wild-type AG protein. It is possible that the truncated protein (containing the MADS-box) can still bind AG target sites. Therefore, the truncated AG may prevent wild-type AG from functioning by competing for target sites. We are in the process of analyzing the effects of other truncated AG proteins.

AGL1 and *AGL2* Are Differentially Expressed during Flower Development

C. Flanagan, H. Ma, Y. Hu

We have examined the expression patterns of two of the MADS-box genes, *AGL1* and *AGL2*, to obtain clues about their functions. RNA dot-blot hybridizations showed that *AGL1* and *AGL2* are both flower-specific genes (Ma et al., *Genes Dev. 5:* 484 [1991]). To characterize the temporal and spatial expression pattern of *AGL1* in more detail, we have used RNA in situ hybridization analysis with gene-specific riboprobes. Our results indicate that *AGL1* expression arises late in flower development (stages 9–10) and is restricted to the carpels. Furthermore, during stages 11 and 12 of flower development (stage 12 is the last stage before the mature bud opens), *AGL1* expression is concentrated in the developing ovules and in the regions of the carpel walls where the two carpels fuse to form the ovary (Fig. 2). Finally, *AGL1* is highly expressed in the nectaries. The *AGL1* expression pattern indicates that *AGL1* is probably involved in the development of the maturing carpel and of ovules.

We have also performed RNA in situ hybridization analysis on wild-type floral sections using an *AGL2*

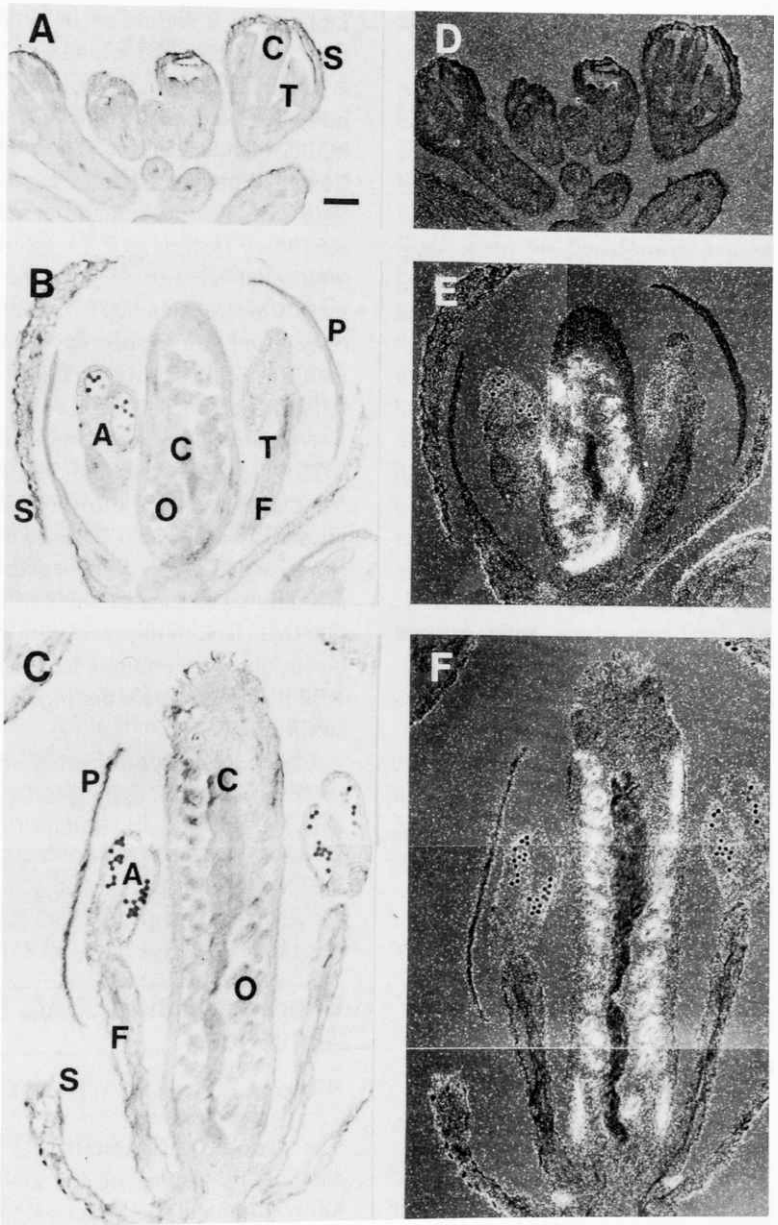

FIGURE 2 RNA in situ analysis of the *AGL1* gene. (*A–C*) Bright field; (*D–F*) dark field. Flowers of stages 5–8 are shown in *A* and *D*, where no *AGL1* expression is detected. At stage 12 (*B* and *E*) and stage 13 (*C* and *F*), *AGL1* is expressed in the ovules. The floral organs are S, sepal; P, petal; T, stamen; A, anther; F, filament; C, carpel; and O, ovule. (Adapted from Fig. 7 of Ma et al., *Genes Dev. 5:* 484 [1991].)

gene-specific riboprobe. *AGL2* expression begins early in flower development (at stage 2), before the onset of *AG* or *AP3* expression but after that of *AP1*, a homeotic gene involved in the transition from inflorescence to floral meristem. *AGL2* expression is ubiquitous and uniformly high in floral primordia and floral organ primordia between stages 2 and 7. However, the expression of *AGL2* becomes organ-specific during later stages of flower development. At stage 8, *AGL2* expression begins to wane in the sepals but remains high in the other organs. Later, during stages 9–10, *AGL2* expression remains high in the carpel,

petals, and (to some extent) filaments, whereas expression is reduced in the sepals and anthers. By stage 12, *AGL2* expression is localized primarily to the ovules. After pollination, the expression of *AGL2* remains detectable in the developing seeds, but it gradually decreases. That *AGL2* expression arises very early in flower development and is expressed in all parts of the young bud is extremely interesting. This pattern is different from the expression patterns of several homeotic genes (e.g., *AG* and *AP3*), which reveal expression in only two of the four floral organ whorls. Only the *AP1* gene is expressed earlier than *AGL2* (at stage 1) and in all cells of floral meristems. However, in stage-3 and later flowers, *AP1*, like the other homeotic genes, is expressed only in two whorls. Thus, the role of the *AGL2* gene product in flower development is likely to be more general than that of the homeotic genes *AG* and *AP3*, and perhaps as important in early flower development as *AP1*, which is required for converting inflorescence meristems to floral meristems.

Additional in situ RNA hybridization experiments with *AGL4*- and *AGL5*-specific probes are in progress. Preliminary results indicate that like *AGL1*, *AGL5* is expressed only in carpels. Further analysis will be done with the *AGL1*, *AGL2*, *AGL4*, and *AGL5* probes.

Expression of the *AGL* Genes in Mutant Flowers

C. Flanagan, H. Ma, Y. Hu

The MADS-box gene family contains homeotic genes (*AG*, *AP1*, *AP3*), as well as a number of other flower-specific genes of unknown function (*AGL*s). Because the sequence of these genes suggests that they are all transcription factors, we think it is likely that they are involved in a regulatory hierarchy controlling flower development. To begin to understand the functional relationship between the homeotic genes and the *AGL*s, we are characterizing the expression pattern of the *AGL* genes in the various homeotic mutant flowers by RNA in situ hybridization analysis.

Analysis of the expression pattern of *AGL2* in an *ag* mutant flower suggests that *AGL2* is not regulated by *AG*. In *ag* mutant flowers, anthers and carpels are replaced by a second set of petals and another flower, respectively. *AGL2* expression arises very early and is ubiquitous in young *ag* buds, as in young wild-type buds. In later-stage *ag* flowers, *AGL2* expression is higher in both sets of petals than in the sepals. The flower primordia of the secondary flower also shows high *AGL2* expression, whereas at later stages of the secondary flower, *AGL2* expression is reduced in the sepals but remains high in the petals, as in the primary flower. Thus, *AGL2* expression is regulated in a stage- and organ-specific manner rather than in a manner specific to the position within the flower (i.e., which whorl).

Northern analysis demonstrated that *AGL1* and *AGL5* are not expressed in *ag* mutant flowers (M. Yanofsky, pers. comm.). Therefore, the interesting possibility arises that these two *AGL* genes are target genes of *AG*. However, because both of these genes are only expressed in the carpels, it is also possible that their lack of expression in *ag* mutants is more indirect, since *ag* mutants lack carpels. Further analysis will be required to distinguish between these possibilities.

In situ RNA hybridization analyses are in progress to determine the expression patterns of *AGL1* and *AGL2* in *ap1* and *ap2* mutant flowers.

Characterization of AG Target Sequences

H. Huang, Y. Mizukami, Y. Hu, H. Ma

The deduced AG amino acid sequence shares substantial similarity with the DNA-binding domains of known transcription factors SRF and MCM1, suggesting that AG is also a transcription factor. To test whether AG is a sequence-specific DNA-binding protein and to determine the sequence(s) of AG target sites, we have performed in vitro DNA-binding studies. To obtain relatively large amounts of AG, we constructed an *Escherichia coli* overexpression plasmid. After screening pools of random oligonucleotides, it was found that the consensus for SRF-binding sites is $CC(A/T)_6GG$ (Pollock and Triesman, *Nucleic Acids Res. 18*: 6197 [1990]). We first tested the ability of AG to bind one of these sequences, CCATTAATGG, using a gel mobility shift assay, and we found AG indeed binds this sequence. As a

control, we performed a parallel binding experiment with the sequence GGATGCATCC, and the result was negative. It has been shown that although SRF and MCM1 can bind to some of each other's natural target sites, they have different spectra of binding sites. The consensus for MCM1 is CC(T/C)$(A/T)_3$NNGG, determined after screening pools of random oligonucleotides (Wynne and Triesman, *Nucleic Acids Res. 20:* 3297 [1992]). To characterize the spectrum of AG target sites, we screened for AG-binding sequences among a pool of random oligonucleotides, essentially as described by Pollock and Treisman. A pool of random single-stranded oligonucleotides each having constant ends of 25 bases and a variable center of 26 bases was converted to double-stranded sequences. The pool of sequences was subjected to binding with AG, and the DNA-protein complexes were separated from unbound DNA using PAGE. The shifted band was cut out and the bound oligonucleotides were eluted for polymerase chain reaction (PCR) amplification. The PCR products were then used for a subsequent round of the AG-binding reaction. After five such rounds, the PCR products were cloned and analyzed by DNA sequencing. From 95 sequences, we find that the AG-binding site sequences are more similar to the MCM1 than the SRF target sequences, with a consensus of $C_{93}C_{91}(A_{51}/T_{33})(A_{60}/T_{21})(A_{68}/T_{25})(T_{59}/A_{29})(T_{40}/A_{26})NG_{78}G_{83}$. Knowing the AG target sequences, we hope to find natural AG target genes in *Arabidopsis thaliana*.

Toward Establishing an Enhancer/Gene Trap Transposon System in *Arabidopsis thaliana*

H. Ma, Y. Hu [in collaboration with V. Sundaresan and R. Martienssen, Cold Spring Harbor Laboratory]

We have continued our experiments to develop an enhancer/gene trap transposon mutagenesis technology, using the maize *Ac/Ds* transposon system. Enhancer/gene trap systems have been successfully used to identify genes with interesting expression patterns and/or phenotypes in *Drosophila* and mice. During the past year, we have worked with Sundar and Rob to characterize the transgenic lines carrying the *Ac* and *Ds* constructs (for more details on the constructs and their introduction into plants by *Agrobacterium*-mediated transformation, see Plant Genetics in last year's Annual Reports from V. Sundaresan and R. Martienssen and from our laboratory). Crosses were done to test the activity of *Ac* in our transgenic lines, the ability of *Ds* elements to transpose, and the feasibility of the Nam/Kan double selection scheme (for a more detailed description, see Plant Genetics in last year's Annual Report). The homozygous lines that have been shown to contain active Ac have been crossed to those carrying either the enhancer-trap or gene-trap Ds. Several hundreds of F1 seeds have been planted and F2 seeds have been collected from them. We will soon select for lines (transposants) that have inherited a transposed Ds and analyze them for expression patterns and mutant phenotypes.

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CELL CYCLE CONTROL IN *SACCHAROMYCES CEREVISIAE*

B. Futcher B. Elliott, I. Fitch, M. Linskens, L. Littlepage, R. Nash, F. Stincone, G. Tokiwa, M. Tyers

Our main interest continues to be the regulation of START in *Saccharomyces cerevisae*. We are most interested in the tethering of division to cell growth and in the generation of the basic cell cycle oscillation. The G_1 cyclin genes *CLN1*, *CLN2*, and *CLN3* encode unstable proteins that form a central part of the machine that measures growth and generates oscillations.

CLN3 Is an Upstream Activator of Other Cyclins

M. Tyers, G. Tokiwa, B. Futcher

We have proposed that *CLN3* helps to activate *CLN1*, *CLN2*, and other G_1 cyclins (Fig. 1). This may occur by activation of the Swi4 and Swi6 transcription factors that are part of a positive feedback loop needed for transcription of *CLN1* and *CLN2*. The "other" G_1 cyclins are collectively called "ClnX" in Figure 1. In the past year, several candidates for the "other" G_1 cyclins have been identified. One is *HCS26*, a cyclin homolog identified (Ogas et al., *Cell 66:* 1015 [1991]) as a suppressor of a *swi4* mutation. Another is *CLB5*, a B-cyclin homolog identified by Epstein and Cross (*Genes Dev. 6:* 1695 [1992]) as a suppressor of *cln1 cln2 cln3* lethality. A third is *ORFD*, an *HCS26* homolog identified fortuitously during the sequencing of another gene. At least two more cyclin homologs have been identified very recently.

A *cln1 cln2 cln3* triple mutant arrests in G_1 phase. We predict from the model (Fig. 1) that in a *cln1 cln2* double mutant, "*CLNX*" activity should be dependent on *CLN3*. We tested this for *HCS26*, *ORFD*, and *CLB5*. A strain with genotype *GAL-CLN3 cln1 cln2* was constructed. This strain is alive on galactose medium, because *CLN3* is expressed from the *GAL* promoter, but is dead on glucose. As shown in Figure 2, this strain expressed *HCS26*, *ORFD*, and *CLB5* only on galactose medium, where *GAL-CLN3* was on. Thus, the prediction was fulfilled, and the dependence of *HCS26*, *ORFD*, and *CLB5* expression on *CLN3* in the *cln1 cln2* background can explain why viability depends on *CLN3*. Interestingly, transcription of *SWI4* also seems to be dependent on the positive feedback loop.

Far1 and Fus3 Link the Mating Pheromone Signal Transduction Pathway to the Cln1, Cln2, and Cln3 Cdc28 Kinase Complexes

M. Tyers, B. Futcher

Cln1, Cln2, and Cln3 are found in complexes with the Cdc28 catalytic subunit, and the complexes have protein kinase activity. The Cln1 and Cln2 complexes coprecipitate proteins of 41, 94, and 116 kD, and these coprecipitated proteins are phosphorylated by the Cln-Cdc28 kinase complex in vitro. The Cln3-Cdc28 complex has a coprecipitated substrate of 45 kD. These coprecipitated substrates are candidates for proteins important in directly mediating START, and so we have made attempts to identify them.

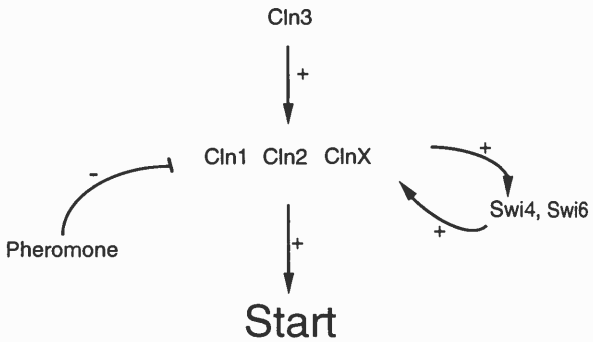

FIGURE 1 Model for activation of START. Cln3 activates Cln1, Cln2, and other "downstream" cyclins, which in turn activate START. Cln3 is weak but constitutive and may activate downstream cyclins through the Swi4 and Swi6 transcription factors. The downstream cyclins then participate in the Swi4,6 positive feedback loop. In the absence of *CLN1* and *CLN2*, *CLN3* is essential because it becomes an essential activator of *HCS26*, *ORFD*, *CLB5*, and perhaps other cyclins (collectively, ClnX). Since *CLN3* is not essential in a wild-type background, there must be *CLN3*-independent mechanisms for transcription of *CLN1* and *CLN2*.

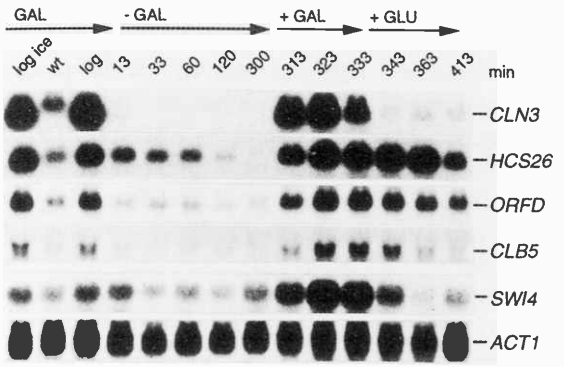

FIGURE 2 *CLN3* is required for transcription of *HCS26*, *ORFD*, and *CLB5* in a *cln1 cln2* strain. A control *CLN1 CLN2 CLN3* strain ("wt") and a *cln1 cln2 GAL-CLN3* strain (all other lanes) were grown in galactose medium to induce the *GAL* promoter. At zero time, the galactose was removed; 300 min later, the cells of the experimental strain had arrested at START, and the *HCS26*, *ORFD*, and *CLB5* transcripts had disappeared (despite the fact that START is the point of maximal transcription of these genes in a wild-type strain). At 303 min, galactose was added back to the culture. The resulting burst of *GAL-CLN3* expression induced expression of the other cyclin genes, resulting in passage through START by about 340 min (by which time, *GAL-CLN3* had been shut off by addition of glucose, which represses the *GAL* promoter). None of these genes are galactose-regulated in a wild-type cell. The actin mRNA is shown as a loading control.

We have identified the 116-kD substrate as the Far1 protein by three criteria. First, when the Cln-Cdc28 complexes were immunoprecipitated and p116 was ^{32}P-labeled using the in vitro kinase reaction, the labeled p116 could be reimmunoprecipitated with an anti-Far1 antibody. Second, when Cln-Cdc28 complexes were immunoprecipitated with an anti-tagged-Cln antibody, Far1 could be detected in the immunoprecipitates with the anti-Far1 antibody. Third, *far1* deletion mutants had no trace of p116.

Far1 is a protein required for α-factor-induced G_1 arrest (Chang and Herskowitz, 1990), and so probably helps inactivate Cln-Cdc28 complexes. When we treated cells with α-factor, the amount of Far1 associated with the Cln1 and Cln2 complexes increased tremendously. This increase was noticeable by 5 minutes of treatment. Shortly thereafter, the amount of Cln1 and Cln2 protein decreased sharply, presumably because of increased turnover, and the Cln1- and Cln2-dependent kinase activities disappeared. α-factor also caused Far1 to associate with Cln3, although in this case, there was no loss of either Cln3 protein or its associated histone H1 kinase activity.

The α-factor-induced association of Far1 with the three Clns was largely dependent on the *FUS3* gene (e.g., Fig. 3). *FUS3* encodes a protein kinase of the MAP kinase family. M. Peter, A. Gartner, G. Ammerer and I. Herskowitz (submitted) have recently shown that Fus3 directly phosphorylates Far1. Thus, a likely chain of events is that α-factor activates Fus3 kinase, which phosphorylates Far1, which then associates with the Cln-Cdc28 complexes, somehow causing proteolysis and inactivation. This is the first clearly traced path between a MAP kinase and a cdc2 kinase.

Proteolysis of Cln3 and the PEST Hypothesis

M. Linskens

THE PEST HYPOTHESIS

The Cln3 protein is very unstable, with a half-life of 2–10 minutes. However, a truncated version of the protein, Cln3-1, which makes cells small and α-factor-resistant, is relatively stable. The stabilized, truncated version lacks the carboxy-terminal third of Cln3. This third contains regions extremely rich in proline, serine, and threonine. These have been called PEST regions and are thought on the basis of their presence in a variety of unstable proteins to be signals for proteolysis (the "PEST hypothesis"). Furthermore, the Cln1 and Cln2 proteins, which are also unstable, also have PEST regions.

To determine whether the PEST regions are important for the instability of Cln3, we made a set of +nested deletions from the 3′ end of the gene. The steady-state abundance of the protein and the protein half-life increase gradually as the PEST regions are removed (Fig. 4). This result is consistent with the PEST hypothesis, as well as with other possibilities. We tried to define the signals for proteolysis more precisely by adding back artificial sequences to stable forms of truncated Cln3. Many different short sequences destabilized the protein, so we were unable to discover any specific signal by this method.

THE LIN PHENOTYPE

The 3′ *CLN3* deletion mutants all ended in unnatural amino acids. In one of the reading frames, the last three amino acids were Leu-Ile-Asn-COOH (LIN). Every one of these LIN alleles behaved as a null allele (none of these alleles are included in Fig. 4). When the frame of the last few bases of the open

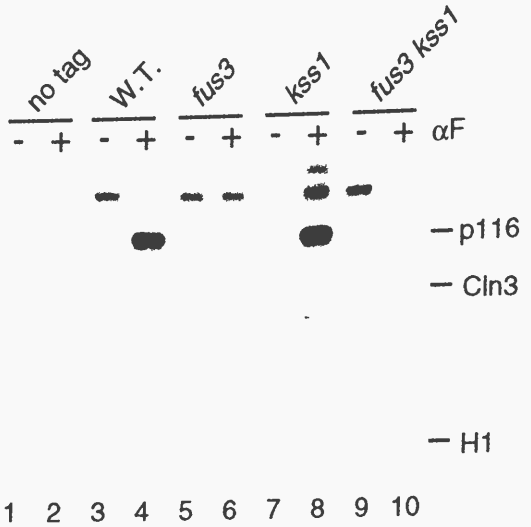

FIGURE 3 α-factor-induced interaction of Far1 with Cln3 depends on *FUS3*. Various wild-type and mutant cultures were grown in the absence or presence of α-factor. Cell extracts were immunoprecipitated with the 12CA5 monoclonal antibody directed against a triple epitope tag at the carboxy terminus of Cln3. An in vitro kinase reaction was carried out with the immunoprecipitated Cln3-Cdc28 complexes, ^{32}P-labeled ATP, and histone H1. Labeled products were separated by SDS-PAGE before autoradiography. The phosphorylated Far1 (p116), Cln3, and histone H1 (H1) bands are indicated. The *KSS1* gene encodes a protein kinase somewhat homologous to that encoded by *FUS3*.

reading frame was shifted to either of the other two frames, yielding amino acids other than LIN, the mutant proteins regained activity. It is as if a carboxy-terminal LIN prevents the protein from working. This could happen if, for instance, LIN efficiently targeted Cln3 to some inappropriate compartment. There is no known yeast protein ending with LIN.

Mechanisms of Heat-shock Resistance

B. Elliott

Although many proteins are induced by heat shocks, it is not clear how these induced proteins are involved in heat-shock resistance. In most cases, mutational inactivation of a heat-shock-induced gene does not cause cellular heat-shock sensitivity. To find out about mechanisms of heat-shock resistance, we looked for mutants that lacked induced heat-shock resistance. So far, two mutants have been extensively characterized and the genes have been cloned. Neither mutant is in the cAMP/Ras pathway, since their phenotypes are unaffected by a wimp background.

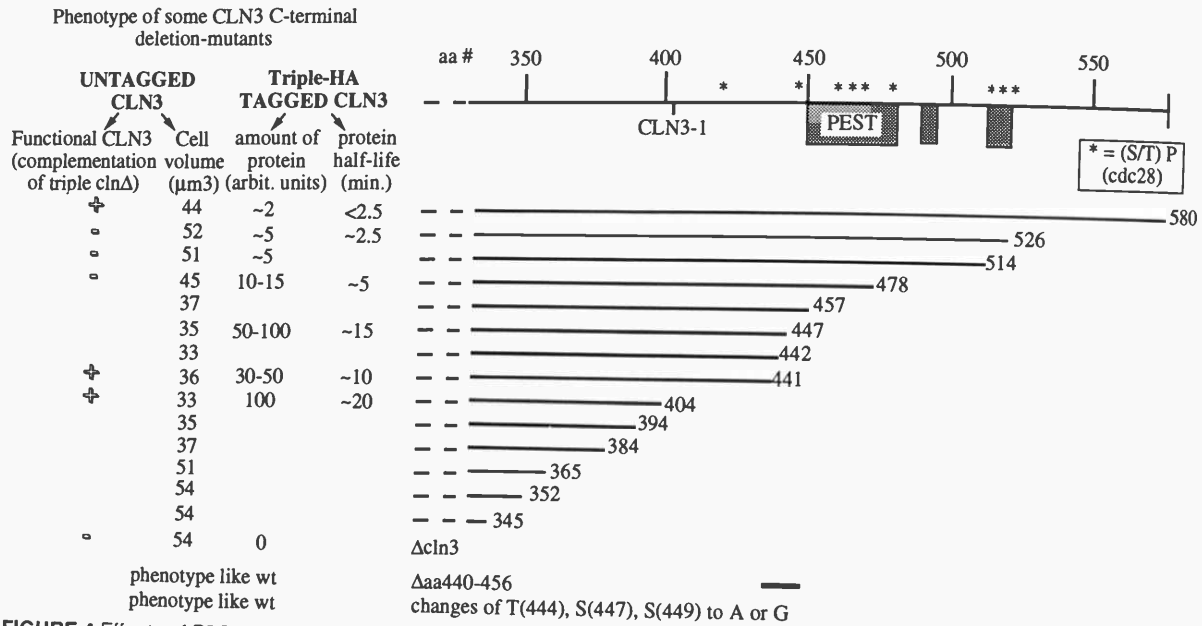

FIGURE 4 Effects of PEST deletions on Cln3 protein half-life. The effects of carboxy-terminal truncations of the Cln3 protein are shown. High levels of Cln3 cause small cell size. Asterisks show positions of potential sites (TP or SP) for phosphorylation by Cdc28. The effects of different truncations on half-life are loosely correlated with the amount of PEST each truncation removes.

One gene, *COX10*, has been previously sequenced. Our *cox10* mutant was partly deficient in stationary-phase-induced heat-shock resistance and also partly deficient in heat-shock-induced heat-shock resistance. The *COX10* gene has previously been implicated in assembly of the cytochrome oxidase complex. Although *cox10* mutants are therefore petite (deficient in respiration), other petite mutants are not deficient in heat-shock resistance. Perhaps the Cox10 protein is a molecular chaperone that helps fold or assemble several proteins, not just cytochrome oxidase, and this is important for induced heat-shock resistance.

The other gene, *HSS1* (heat-shock-sensitive), has not been previously sequenced. The *hss1* mutant was totally deficient for induced heat-shock resistance. The gene is somewhat homologous to the *CIF1/FDP1* gene of yeast. *CIF1/FDP1* is thought to encode a subunit of trehalose-6-phosphate synthase, and *cif1/fdp1* mutants are heat-shock-sensitive. It may be that *HSS1* is also involved in trehalose synthesis. In yeast, high levels of intracellular trehalose are extremely well correlated with high levels of heat-shock resistance. This may mean that trehalose is a primary cause of heat-shock resistance. Alternatively, it may mean that regulation of trehalose synthesis is intimately linked to regulation of heat-shock resistance. New mutant alleles of *HSS1* along with measurements of trehalose content may distinguish these possibilities.

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TRANSCRIPTION AND CELL CYCLE REGULATION IN YEAST

K.T. Arndt A. Sutton, C. Devlin, C. DiComo, A. Doseff, M.J. Fernandez-Sarabia, F. Lin, M. Luke, T. Zhong

We are using the yeast *Saccharomyces cerevisiae* to investigate two basic cellular processes: regulation of transcription by RNA polymerase II and regulation of commitment for entry into the cell cycle.

Genetic Selection Schemes for General Transcription Factors

C. Devlin

In the yeast *Saccharomyces cerevisiae*, three proteins, BAS1, BAS2, and GCN4, bind to the *HIS4* promoter to activate transcription of the *HIS4* gene. A strain containing deletions of *BAS1*, *BAS2*, and *GCN4* has a *His*$^-$ phenotype due to insufficient transcription of *HIS4*. An additional protein, RAP1, also binds to the *HIS4* promoter but is not by itself able to activate transcription of *HIS4*. At *HIS4*, RAP1 functions to phase nucleosomes away from the GCN4-, BAS1-, and BAS2-binding sites so that these sites are accessible when present within chromatin. Our research objectives with *HIS4* can be divided into two main areas: One area studies the details of how BAS1, BAS2, GCN4, and RAP1 function for activation of *HIS4* transcription. The second area of research uses

the *HIS4* promoter for genetic selection schemes to isolate mutations in genes whose products function in transcription. To identify mutations in genes encoding general transcription factors, we isolated *His*$^+$ revertants of a strain containing deletions of *BAS1*, *BAS2*, and *GCN4*. From these studies, we obtained the *sit1* through *sit12* mutations. The *sit* mutations cause a strong growth defect and therefore occur in genes required for normal cellular growth. So far, the *sit* mutations have altered either RNA polymerase II (*sit1* or *sit2*), nucleosomes (*sit5*), and a factor (*sit4*) that may regulate a general transcription factor or chromatin.

We are presently analyzing the *sit7* and *sit10* mutations for their global transcriptional alterations and have cloned the corresponding wild-type *SIT7* and *SIT10* genes.

SIT4 Protein Phosphatase and Regulation of G_1 Cyclins

M.J. Fernandez-Sarabia, C. DiComo, A. Sutton

The *SIT4* gene predicts a protein that is 55% identical to the mammalian type-2A and 43% identical to the mammalian type-1 protein phosphatase catalytic subunits. However, the SIT4 protein phosphatase is a unique phosphatase (distinct from the *S. cerevisiae* type-1 and type-2A phosphatases) that is required in late G_1 for passage through START. START is a place in the late G_1 phase of the cell cycle where nutrients and mating signals exert control over entry into the cell division cycle. Once cells have passed (or "executed") START, they are committed for DNA synthesis and cell division.

SIT4 coimmunoprecipitates with two high-molecular-mass proteins, termed p155 and p190. The association of SIT4 with p155 and p190 is cell-cycle-dependent. In G_1 daughter cells, SIT4 is not associated with p155 and p190. At a time very close to (or at) START, SIT4 associates in separate complexes with p155 and p190. SIT4 remains associated with p155 and p190 until about the middle or end of mitosis. Therefore, SIT4 associates with p155 and p190 very close to the time that SIT4 executes its function. In our model, p155 and p190 proteins are regulatory subunits of SIT4 that target SIT4 to the proper substrates or regulate the substrate specificity of SIT4.

TRANSCRIPTION OF THE G_1 CYCLINS

Because SIT4 is required for the execution of START, we examined whether other known proteins required for the execution of START are altered in *sit4* mutants. Known proteins that are required for the execution of START are the G_1 cyclins and the CDC28 kinase. The G_1 cyclins associate with the CDC28 kinase catalytic subunit. In other organisms, there is some evidence that cyclins are required for the kinase activity of cdc2 (equivalent to CDC28 of budding yeast). In budding yeast, the G_1 cyclins fall into two groups. Group 1 is composed of the *SWI4*-dependent G_1 cyclins *CLN1*, *CLN2*, and *HCS26*. The mRNA and protein levels of these G_1 cyclins vary greatly during the cell cycle. In early G_1, the mRNA and protein levels of the group-1 cyclins are very low. During G_1 (especially mid and late G_1), the mRNA levels of the group-1 cyclins increase at a nearly exponential rate, reaching a maximal level in late G_1, at a time very close to (or at) START. After START has been executed, the mRNA and protein levels of the group-1 cyclins rapidly decrease. Transcription of the group-1 G_1 cyclin genes requires the SWI4 transcription factor, which binds to the promoter of group-1 cyclin genes. G_1 cyclin group 2 has only one known member: CLN3. Although *CLN3* mRNA levels vary somewhat during the cell cycle, *CLN3* mRNA (and possibly CLN3 protein) is present throughout the cell cycle. In addition, transcription of *CLN3* is not *SWI4*-dependent.

Many experiments in our laboratory have shown that the SIT4 protein phosphatase is required for the normal accumulation of the SWI4-dependent group-1 cyclin mRNAs (*CLN1*, *CLN2*, and *HCS26*) during late G_1. In contrast, SIT4 is not required for the transcription of *CLN3*. A model for the role of SIT4 for the transcription of *CLN1*, *CLN2*, and *HCS26* is shown in Figure 1. At some time during late G_1, the levels of *CLN1* and *CLN2* RNAs increase dramatically. This late G_1 increase in the levels of *CLN1* and *CLN2* RNAs requires at least one functional *CLN* gene (either *CLN1*, *CLN2*, or *CLN3*) and is stimulated by the hyperactive *CLN3-1* or *CLN3-2* mutation. In addition, the late G_1 increase in the levels of *CLN1* and *CLN2* RNAs requires the CDC28 kinase. These results (from the laboratories of F. Cross and K. Nasmyth) suggested that the *CLN* genes and *CDC28* jointly function via a positive feedback loop for stimulating the late G_1 accumulation of *CLN1* and *CLN2* RNAs. The targets of the CLN1/CDC28, CLN2/CDC28, and CLN3/CDC28 kinases in this

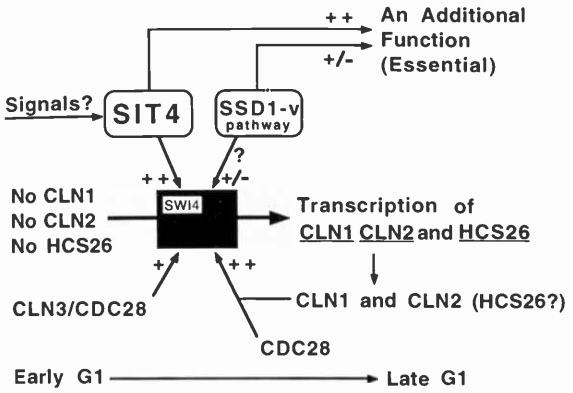

FIGURE 1 Model for SIT4 function in late G_1.

feedback loop are not known and are included in the black box. These substrates could be the CLN/CDC28 complexes themselves, the SWI4 and SWI6 transcription factors, and/or some other currently unknown factor. In Figure 1, the arrow for SIT4 function points toward SWI4 within the black box because SIT4 is required for the accumulation of *CLN1*, *CLN2*, and *HCS26* RNAs at least partly because SIT4 is required for the accumulation of *SWI4* RNA. In fact, the mechanism giving rise to the increase in *SWI4* mRNA levels may be part of the same positive feedback loop that gives increased transcription of *CLN1*, *CLN2*, and *HCS26* RNAs. The *CLN3* pathway and the *CLN1*, *CLN2*, *HSC26* pathways for stimulation of *CLN1*, *CLN2*, *HSC26* transcription may also function via *SWI4*.

Since *SIT4* is required for *SWI4* function, our model predicts that *sit4* mutations should behave like *swi4* mutations (at least for certain functions). A number of tests of this model have been made, all of which support the model. One test is the following: A cell can survive on CLN1 (in the absence of CLN2 and CLN3), on CLN2 (in the absence of CLN1 and CLN3), or on CLN3 (in the absence of CLN1 and CLN2). However, a cell cannot survive in the absence of CLN1, CLN2, and CLN3. For this reason, deletion of *SWI4* (gives lower levels of *CLN1* and *CLN2* mRNAs) combined with a deletion of *CLN3* results in an extremely slow growth rate. Our model predicts that *sit4* mutants should also be more dependent on the function of *CLN3*, whose transcription does not depend on *SIT4*. That *sit4* Δ*cln3* cells are almost inviable supports the model. Therefore, in this genetic test (near inviability in combination with deletion of *CLN3*), *sit4* behaves like *swi4* mutants. A further prediction of the model is that if the *CLN2*-coding sequences are transcribed from a promoter that does not depend on *SIT4* (or *SWI4*), then *CLN3* should be dispensable in a *sit4* mutant strain. These experiments show that the requirement of a *sit4* mutant for *CLN3* is completely eliminated if CLN2 is expressed from a promoter that is not *SIT4* (or *SWI4*)-dependent. Moreover, the requirement of a *sit4* mutant for *CLN3* is completely eliminated if *SWI4*-coding sequences are expressed from a promoter that is not *SIT4*-dependent Other tests also suggest that the requirement for *SIT4* in transcription of *CLN1*, *CLN2*, and *HCS26* is at least partly via the requirement for *SIT4* in the expression of *SWI4*. The mechanism that determines when, and the rate at which, the RNA levels of *CLN1*, *CLN2*, and *HCS26* (and *SWI4*) increase during G_1 is central to the cell's decision to enter the cell division cycle. That SIT4 is required for this mechanism and that SIT4 associates with p155 and p190 at a point in late G_1 very close to START suggest that the SIT4 phosphatase is part of the mechanism that regulates the timing of START.

DESTRUCTION OF THE G_1 CYCLINS

After START has been executed, the mRNA and protein levels of *CLN1* and *CLN2* decrease dramatically. Interestingly, not only is SIT4 required for the normal expression of *CLN1*, *CLN2*, and *HCS26*, but it is also required for the normal disappearance of CLN2 (and possibly CLN1 and HCS26). Normally, at the nonpermissive temperature, *sit4* mutants arrest in late G_1 with very low levels of *CLN1* and *CLN2* (mRNA or protein) and with a 1n DNA content. This is because SIT4 is required for the late G_1 transcription of *CLN1* and *CLN2* and the execution of START. However, if CLN2 is expressed from a promoter that is SIT4-independent, then the *sit4* mutants arrest with high levels of CLN2 and with a 2n DNA content. Therefore, the requirement for SIT4 for the initiation of DNA synthesis is via the requirement for SIT4 for expression of CLN2 (and probably CLN1).

The CLN2 (expressed from a non-SIT4-dependent promoter) present in the *sit4*-arrested cells is hyperphosphorylated and has a high associated kinase activity. When these *sit4*-arrested cells are released from the *sit4* block (by returning to the permissive temperature), the CLN2-associated kinase activity decreases rapidly, followed very closely by the disappearance of the CLN2 protein. In vitro, the SIT4 PPase not only dephosphorylates CLN2, but also mediates the destruction of the CLN2 present in

CLN2 immunoprecipitates (as assayed by Western analysis of the immunoprecipitates for CLN2). The SIT4-mediated destruction of CLN2 in vitro requires the phosphatase activity of SIT4, because addition of okadaic acid to the reactions prevents the destruction of CLN2. In addition, the SIT4-mediated destruction of CLN2 requires that CLN2 have a particular phosphorylation state. Pretreatment of the CLN2 with a nonspecific phosphatase removes all of the phosphates of CLN2 (and CLN2 remains stable after this treatment). Moreover, subsequent addition of SIT4 to the fully dephosphorylated CLN2 causes no destruction of the CLN2.

The results presented above indicate that CLN2 is probably a direct substrate of SIT4. Presently, we do not know if the protease that destroys CLN2 comes from the added SIT4 or if SIT4 activates a protease that coimmunoprecipitates with CLN2. Our current studies are designed to identify the phosphoserine or phosphothreonine residue on CLN2 that SIT4 dephosphorylates and to examine the in vivo consequences (stability, kinase activity, and the effects on START) of alterations of this residue.

Our results raise the possibility that SIT4 controls two aspects of CLN2 function. First, SIT4 could regulate the rate of increase of *CLN2* RNA and control the timing of START. Second, after START has been executed, SIT4 could regulate the disappearance of the CLN2/CDC28 kinase via the destruction of CLN2. The cells may require inactivation of the CLN2 kinase for normal cell cycle progression and that this process correspond to a late G_1 check point. Since SIT4/p155 and SIT4/p190 most likely regulate different processes, future experiments with SIT4 will require reagents so that the specific functions of SIT4/p155 and SIT4/p190 can be separated and identified. One form of SIT4 could regulate transcription of *CLN2*, and the other form of SIT4 could regulate destruction of *CLN2*.

Analysis of the Role of the SSD1 Protein in the Yeast Cell Cycle

A. Sutton

In certain strain backgrounds, deletion of *SIT4* is lethal, and temperature-sensitive *sit4* strains arrest in late G_1 at the nonpermissive temperature. However, in other strain backgrounds, deletion of *SIT4* results in viability, although the strains grow very slowly. The viability or inviability of a strain containing a deletion of *SIT4* is due to a single unlinked genetic locus, termed *SSD1*. Alleles of *SSD1* that result in viability in combination with a deletion of *SIT4* are called *SSD1-v* alleles; alleles that result in death when combined with a deletion of *SIT4* are called *ssd1-d* alleles. Deletion of *SSD1* by itself results in only subtle phenotypic effects, but many lines of genetic evidence implicate SSD1 in G_1 regulation. The allele of *SSD1* that suppresses the lethality caused by deletion of *SIT4* has recently been found to also suppress phenotypic defects caused by mutations in a wide variety of pathways. For example, *SSD1-v* is able to suppress the lethality of a strain containing deletions of the *CLN1* and *CLN2* genes, the heat-shock sensitivity of strains containing mutations in the RAS/cAMP pathway, the temperature sensitivity of strains containing mutations in the protein kinase C pathway, and the temperature sensitivity of strains containing mutations in subunits of RNA polymerase C (III). There is no obvious connection among these diverse mutations. However, in each pathway, phosphorylation and dephosphorylation play key regulatory roles. Therefore, the possibility exists that SSD1 functions by interacting with either a phosphatase or kinase common to all of the above pathways. To address this, we have looked for phosphatase and kinase activities in immunoprecipitates of SSD1. To date, we have been unable to detect either activity associated with SSD1. However, such activities may be very weak, as the levels of *SSD1* transcript and protein in the cell are very low.

To help elucidate the cellular role of SSD1, we recently set up a genetic screen to identify other genes whose products function in the SSD1 pathway. In a strain containing *ssd1-d*, *SIT4* is essential. In an *SSD1-v* strain, loss of SIT4 function does not cause lethality. We created a strain in which *SIT4*-coding sequences are under the control of the *GAL10* promoter (*pGAL-SIT4)* and which has an *SSD1-v* allele. When grown on galactose medium, this strain grows very well because *SIT4* is expressed. When grown on glucose medium, *SIT4* is not expressed, but the strain is viable because of *SSD1-v*. If either *SSD1-v* or any gene required for *SSD1-v* function is mutated, then this strain will be alive on galactose but dead on glucose. We mutagenized the *pGAL-SIT4 SSD1-v* strain with ethylmethanesulfonate (EMS) and looked for mutants that are alive on galactose but dead on glucose. As predicted, we obtained mutations in *SSD1-v*. In addition, we obtained an additional

nine mutations that fall into eight complementation groups (therefore, we have not saturated the genome). The wild-type genes for these mutations are being cloned by their ability to rescue the lethality on glucose. These genes are called *PDL1-8* (for phosphatase-deficient lethality). The *pdl1* mutation lies in a previously identified gene, *CHS1*. *CHS1* encodes a chitin synthase enzyme and is believed to be required for repair of the cell wall after budding. We have some evidence from other studies that implicates both *SSD1-v* and *SIT4* in the budding process, and isolation of *CHS1* as a synthetic lethal with *SIT4* provides additional support for this. *PDL2* and *PDL3* have been cloned, and sequence analysis of these clones as well as the cloning of additional *PDL* genes is under way. We hope that the analysis of these genes will allow us to determine the function of SSD1. The results from this approach may also provide insight into the role of the SIT4 phosphatase in the cell cycle.

A Genetic Search for the SIT4 Regulatory Subunits

A. Doseff

In an otherwise wild-type cell, the *SSD1* gene is dispensable. Cells containing a deletion of *SSD1* have a growth rate that is very similar to that of wild-type cells. However, in cells containing a deletion of *SSD1*, the *SIT4* gene is essential. Put another way, Δ*sit4* strains require *SSD1-v*. Therefore, to obtain mutations in genes functioning in the *SIT4* pathway, we isolated mutants that require the *SSD1-v1* gene for viability. We are most interested in obtaining the genes encoding p190 and p155, two proteins that bind to SIT4.

From a screen of 160,000 colonies resulting from EMS-mutagenized cells, we obtained about 80 mutants that are inviable or extremely slow growing in the absence of the *SSD1-v1* gene. As expected, some of these mutants contain a mutation in the *SIT4* gene itself and confirm the rationale of our screen. The non-*sit4* mutants were placed into six main complementation groups, termed *sap1* through *sap6*. These *sap* mutations cause a slow-growth and/or temperature-sensitive phenotype. *SAP1* is a previously unidentified gene whose protein product has no homology with proteins in the current database. *SAP2* and *SAP3* are previously identified genes, termed *SMP3* and *SLK1* (also known as *BCK1*), respectively, that function in the protein kinase C pathway. For this reason, we are currently examining the relationship of SIT4 to the protein kinase C pathway. The *sap6* mutations are really mutations in the *SAP3* gene that give rise to intragenic complementation with our *sap3* mutations. The *SAP4* and *SAP5* genes have been cloned and are under analysis. Hopefully, the analysis of the *sap* mutants will provide us with every gene whose product is required for the *SIT4* pathway (or at least those functions of SIT4 that are essential in the absence of the *SSD1-v* gene). The identification of the genes for p155 and p190 will greatly aid in the biochemical analysis of SIT4.

Role of Type-2A Protein Phosphatases in the *S. cerevisiae* Cell Cycle

F. Lin

S. cerevisiae has two genes, *PPH2*α and *PPH2*β, that encode good homologs (about 80% identity) of the catalytic subunit of mammalian type-2A protein phosphatases (PPase). One of our goals is to determine the role(s) of the *S. cerevisiae* type-2A phosphatases in the cell cycle. This analysis is greatly simplified with strains containing temperature-sensitive mutations in *PPH2*α or *PPH2*β. Since we had a very good temperature-sensitive mutation in *SIT4* (i.e., *sit4-102*) and since the amino acid residue altered in the SIT4-102 protein is conserved between SIT4 and all known type-2A PPases, we prepared *PPH2*α and *PPH2*β genes that alter this residue. Strains containing either the *pph2*α-102 or *pph2*β-102 genes as the only source of type-2A PPase are temperature sensitive for growth. When an asynchronous culture of either strain is shifted to 37°C, greater than 90% of the cells arrest with a 2n DNA content. The majority of the cells contain a single nucleus with a *single* microtubule-organizing center. These results suggest that type-2A phosphatases are required for spindle pole body duplication or separation but not for DNA synthesis. Since the CDC28 kinase is required for spindle pole body duplication and progression from the G_2 phase into mitosis, we analyzed whether the activity of the CDC28 kinase is dependent on the type-2A phosphatase activity.

These studies show that when the type-2A PPase mutants are shifted to the nonpermissive temperature, the histone H1 kinase activity of CDC28 rapidly (within a few minutes) decreases to very low levels. The CDC28 kinase seems to be active only when it is associated with cyclins. For spindly pole body separation and for entry into mitosis, CDC28 associates with the B-type cyclins CLB1, CLB2, CLB3, and CLB4. We have found that a particular phosphorylated form of CLB is present in lower amounts in the absence of type-2A PPase function. These results raise the possibility that type-2A PPases are required (directly or indirectly) for the activation of the kinase activity of the CDC28/CLB2 complex via the modulation of the phosphorylation state of CLB2. Our current experiments are aimed at testing this possibility.

The SIS1 Protein: An Essential dnaJ Homolog

M. Luke, T. Zhong

The budding yeast *SIS1* gene encodes the only full-length dnaJ homolog known to be essential for viability. The *Escherichia coli* dnaJ protein is a heat-shock protein required for bacteriophage λ, P1 phage, and normal host DNA replication. Although it is believed that the mechanism by which dnaJ functions is to mediate the dissociation of specific protein/protein complexes, the precise cellular function provided by dnaJ is not known.

Like dnaJ, SIS1 is a heat-shock protein. To determine the essential cellular function provided by SIS1, we isolated extragenic suppressors of the temperature-sensitive phenotype resulting from the *sis1-85* mutation. These experiments show which mutations that alter the levels or structure of 60S ribosomal subunits suppress the temperature-sensitive phenotype of *sis1-85* strains. These results led us to determine whether SIS1 is required for translation. At the nonpermissive temperature, temperature-sensitive *sis1-85* and *sis1-86* strains rapidly accumulate high levels of 80S ribosomes and have decreased levels of polysomes. These results indicate that SIS1 is required for the initiation of translation. At the nonpermissive temperature, elongation can continue in the absence of new rounds of initiation (which results in decreased levels of polysomes). The block in new rounds of initiation results in the accumulation of high levels of inactive 80S ribosomes. Additional experiments (effects of cycloheximide, high salt, etc.) confirm these conclusions. Moreover, mutations that alter the structure or levels of 60S ribosomal subunits suppress the temperature-sensitive phenotype of *sis1* strains by eliminating the block in translation initiation. That SIS1 is required for the initiation of translation raises the possibility that SIS1 might associate with ribosomes. Direct biochemical analysis shows that SIS1 associates with certain forms of ribosomes. In summary, these experiments show that the SIS1 heat-shock protein is required for translation, a fundamental cellular process. Future experiments will be directed toward understanding the precise translational initiation step that requires SIS1.

Parallel experiments show that a fraction of the SIS1 protein associates in a very large molecular weight complex with a 40-kD protein (which we term p40). We have purified p40 (via its association with SIS1) and have obtained the amino acid sequence of three different peptides (in collaboration with R. Kobayashi). The biochemical and molecular analyses of p40 are in progress.

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INITIATION AND TERMINATION OF TRANSCRIPTION IN HUMAN snRNA GENES AND HIV-1

N. Hernandez R.W. Henry S.M. Lobo R. Mital F.C. Pessler C.L. Sadowski M. Sheldon M.L. Sullivan

SMALL NUCLEAR RNA GENES

In eukaryotes, transcription is carried out by three different RNA polymerases, none of which can recognize its target promoters directly. Instead, promoter elements are first recognized by specific transcription factors that then recruit the correct RNA polymerase. Because RNA polymerase I, II, and III promoters are generally very different in structure, it has long been assumed that RNA polymerase specificity is achieved through the binding of very distinct sets of transcription factors. However, the promoters of human small nuclear RNA (snRNA) genes are very similar in structure even though some of them are recognized by RNA polymerase II and others are recognized by RNA polymerase III. This suggests that they bind identical transcription factors and raises the question of how RNA polymerase specificity is achieved.

The RNA polymerase II U1 and U2 snRNA promoters and the RNA polymerase III U6 promoter contain two interchangeable elements: The distal sequence element (DSE), which is characterized by the presence of an octamer motif and functions as an enhancer of transcription, and the proximal sequence element (PSE), which is essential for transcription. The U6 promoter contains in addition a TATA box that determines RNA polymerase III specificity. When the TATA box is mutated, the U6 promoter becomes a predominantly RNA polymerase II promoter, and when the TATA box is inserted into the U2 promoter, the U2 promoter becomes a predominantly RNA polymerase III promoter.

The U6 promoter is very different from the gene internal RNA polymerase III promoters characterized previously. Thus, the promoters of the 5S genes consist of an internal control element (ICR) and the promoters of tRNA genes, *Alu* sequences, and several viral genes, including the adenovirus-2 VAI and VAII genes, consist of an A and B box. The factors required for transcription of the 5S and tRNA-type RNA polymerase III genes were fractionated more than 10 years ago over phosphocellulose into three fractions referred to as the PC-A, PC-B, and PC-C fractions, which contain TFIIIA, TFIIIB, and TFIIIC, respectively (see Fig. 1). TFIIIA binds to the ICR of the 5S genes, and this event allows the subsequent recruitment of TFIIIC, TFIIIB, and RNA polymerase III into the initiation complex. tRNA-type genes do not require TFIIIA for transcription, because TFIIIC can bind directly to the A and B boxes and recruit TFIIIB and RNA polymerase III. TFIIIB is thought to contact RNA polymerase III directly and thus to be involved in transcription of all RNA polymerase III genes. What is known about the composition of these three factors? TFIIIA is a zinc finger protein whose corresponding cDNA has been cloned. TFIIIB and TFIIIC have been purified from yeast and human cells. TFIIIB has been reported to consist of a single 60-kD polypeptide or to contain at least two polypeptides of 70 and 90 kD. TFIIIC consists of at least five polypetides.

In the last year, we have continued our studies of the transcription factors involved in U1, U2, and U6 transcription, and we have compared the factors required for RNA polymerase III transcription of the U6 gene with those required for transcription of other RNA polymerase III genes. Specifically, we have initiated the characterization of the TFIIIB fraction and the factor that recognizes the U1, U2, and U6 PSEs. These studies have revealed that the TATA box binding protein (TBP), first thought to be involved only in transcription of TATA-containing RNA polymerase II mRNA genes, in fact also participates in the initiation complexes of RNA polymerase II snRNA genes and RNA polymerase III genes.

HUMAN IMMUNODEFICIENCY VIRUS

Expression from the human immunodeficiency virus type 1 (HIV-1) is regulated in part by a viral protein termed Tat. Tat is a strong activator of HIV-1 gene expression and acts through an RNA target, TAR, en-

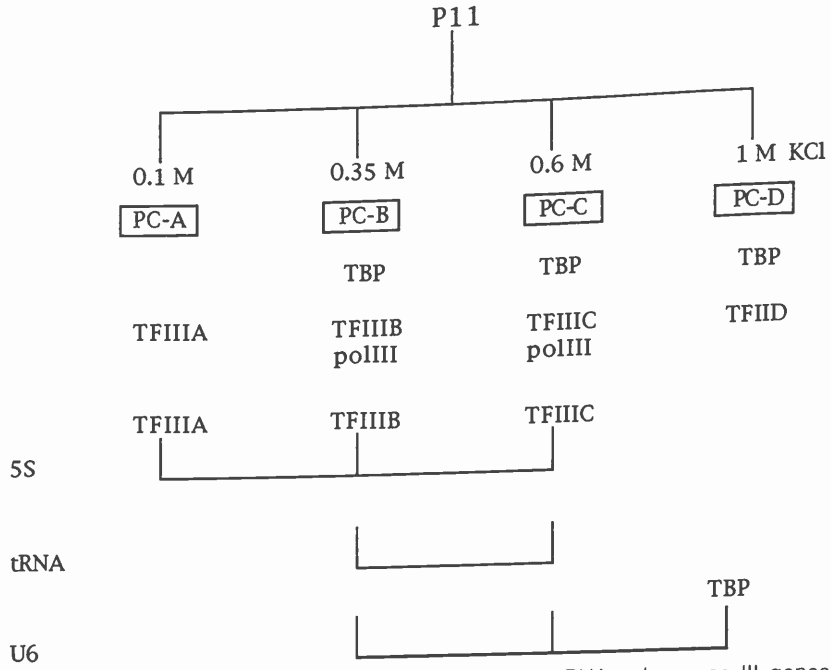

FIGURE 1 Factors required for transcription of different RNA polymerase III genes. The fractionation of the TFIIIA, TFIIIB, and TFIIIC factors on a phosphocellulose column is indicated. The PC-B, PC-C, and PC-D fractions contain TBP as determined by immunoblotting.

coded between positions +18 and +44 downstream from the transcriptional start site. TAR corresponds to the upper part of a stable stem-and-loop structure assumed by the first 59 nucleotides of HIV-1 RNA. The mechanism by which Tat activates transcription is controversial. Tat has been reported to enhance initiation of transcription, elongation of transcription, or both. The proposal that Tat stimulates transcription elongation stems in part from the observation that in the absence of Tat, the HIV-1 promoter synthesizes a large number of short transcripts, whereas in the presence of Tat, the number of short transcripts decreases with a concomitant increase in the number of full-length transcripts.

We are studying an element, the inducer of short transcripts or IST, that is located entirely between positions −5 and +82 relative to the HIV-1 start site. This element has very unusual properties: It activates transcription from the HIV-1 promoter and other promoters very strongly, but the resulting RNAs are prematurely terminated around position +60. Thus, IST is responsible for the formation of the short transcripts observed in the absence of Tat and seems to direct the formation of transcription complexes that are unable to elongate efficiently. We have now localized and characterized more precisely the IST element by mutational analysis. Some of these mutations debilitate IST but leave TAR intact. Importantly, the constructs carrying these mutations can still be very efficiently *trans*-activated by Tat, even though they do not make any detectable short transcripts. This indicates that the short transcripts are not required for Tat *trans*-activation and therefore that Tat does not function by allowing elongation of the short transcripts. Thus, whereas IST activates the formation of transcription complexes unable to elongate efficiently, Tat appears to activate the formation of conventional initiation complexes that are capable of efficient elongation. In other words, Tat acts at the level of initiation.

Components of the TFIIIB Fraction Involved in Transcription of Different Types of RNA Polymerase III Genes

S.M. Lobo, M.L. Sullivan, N. Hernandez

We have shown before that as illustrated in Figure 1, transcription of the U6 gene requires the PC-B, PC-

C, and PC-D fractions. The PC-D fraction is not required for transcription of 5S or tRNA-type genes. It is required, however, for transcription of RNA polymerase II mRNA genes because it contains TFIID, a complex composed of TBP and associated proteins that recognizes the TATA box of mRNA promoters. Like that for basal RNA polymerase II transcription from mRNA promoters, the PC-D fraction can be replaced by recombinant human TBP for transcription of the RNA polymerase III U6 gene. Furthermore, TBP binds to the U6 TATA box but not to mutated TATA boxes that switch the specificity of the U6 promoter to RNA polymerase II. This observation indicates that in the U6 promoter, binding of TBP to the U6 TATA box determines transcription by RNA polymerase III.

Figure 1 shows that the TBP is present not only in the PC-D fraction, but also in the PC-B and PC-C fractions. In fact, nearly 50% of the cellular TBP fractionates in the PC-B fraction, whereas a much smaller amount is found in the PC-C fraction. This observation raises several questions. Why must the PC-B and PC-C fractions be supplemented with TBP for reconstitution of U6 transcription? Is the TBP present in the PC-B and PC-D fractions modified such that it is inactive for U6 transcription? And what are the functions of TBP in the PC-B and PC-C fractions? To address these points, we used a panel of anti-TBP monoclonal antibodies that we had generated previously and tested their effect on transcription from the U6 and VAI gene. We found that, as expected, addition of anti-TBP antibodies to an extract inhibits U6 transcription. Interestingly, it also inhibits transcription from the 5S and VAI genes, suggesting that TBP is required for transcription of all RNA polymerase III genes. Transcription of the 5S and VAI genes requires the PC-B and PC-C fractions, which both contain TBP (Fig. 1). We determined that the TBP in the PC-C fraction can be removed by passage over an anti-TBP column without affecting VAI transcription. Thus, the TBP required for VAI transcription is provided by the PC-B fraction.

To characterize this fraction better, we fractionated it by chromatography over Mono-Q and found that the TFIIIB activity separates into two components, eluting at 0.38 M (0.38 M-TFIIIB) and 0.48 M (0.48 M-TFIIIB) KCl, respectively. Both of these components are required to reconstitute VAI transcription. We then showed that the active component of the 0.38 M-TFIIIB fraction corresponds to a TBP-containing complex, which cannot be replaced by TBP for transcription of the VAI gene. In sharp contrast, U6 transcription does not require the 0.38 M-TFIIIB component. Together, these results indicate that although all RNA polymerase III genes use TBP for transcription, they differ in the specific form of TBP required. TATA-containing RNA polymerase III genes such as the U6 gene use TBP or perhaps the TFIID complex, whereas TATA-less RNA polymerase III genes such as the VAI gene require the 0.38 M-TFIIIB complex. This suggests that at least one of the roles of the polypetides associated with TBP in the 0.38 M-TFIIIB complex is to anchor TBP to the initiation complex. These results also reveal that TFIIIB is a complex factor composed of several subunits. We are now characterizing further the TBP-associated proteins in the 0.38 M-TFIIIB complex.

Purification of the 0.48 M-TFIIIB Activity

R. Mital, N. Hernandez

Unlike 0.38 M-TFIIIB, which is required for transcription of TATA-less but not TATA-containing RNA polymerase III genes, the 0.48 M-TFIIIB fraction is required for transcription of all RNA polymerase III genes. However, this fraction is very crude, and it is therefore possible that the component(s) involved in U6 transcription differs from those involved in transcription of the VAI gene. To identify and characterize the active components of this fraction, we have started their purification.

Factors Involved in Transcription of the Human U1 and U2 snRNA Genes

C.L. Sadowski, R. W. Henry, S.M. Lobo, N. Hernandez

Unlike RNA polymerase II transcription of mRNA genes and RNA polymerase III transcription of the U6 gene, RNA polymerase II transcription of snRNA genes is difficult to reproduce in vitro, and this has so far prevented the biochemical characterization of the factors involved in transcription of these genes. Following a protocol developed by others (Gunderson et

al., *Genes Dev. 4:* 2048 [1990]), we have obtained accurate and efficient initiation of transcription of the human U1 and U2 genes in vitro. Using this system, we have examined whether RNA polymerase II transcription from the U1 and U2 genes requires TBP. Since these genes do not have a TATA box in their promoters, and since the binding of TBP to the U6 TATA box results in RNA polymerase III transcription, it is conceivable that U1 and U2 transcription does not use TBP. However, we find that depletion of extract with several monoclonal anti-TBP antibodies, but not with nonspecific monoclonal antibodies, inhibits transcription from these genes. Interestingly, transcription cannot be restored by addition of recombinant TBP or by addition of any of the known TBP-containing complexes, 0.38 M-TFIIIB and TFIID. Instead, transcription is restored by addition of fractions enriched in a factor that binds to the PSEs of the U1, U2, and U6 promoters. This suggests that this factor is a TBP-containing complex. Indeed, in an electrophoretic mobility shift assay with a PSE-containing probe, addition of anti-TBP monoclonal antibodies disrupts the PSE-binding factor complex. These results identify a novel TBP-containing complex that may be involved in transcription of both RNA polymerase II and RNA polymerase III snRNA genes. These results also suggest that the U6 initiation complex contains two molecules of TBP, one bound to the TATA box and the other to the PSE as part of the PSE binding complex.

Characterization and Purification of the PSE-binding Factor

R.W. Henry, C.L. Sadowski, M.L. Sullivan, N. Hernandez

The PSE-binding factor is of great interest to us, because as described above, it may be involved in RNA polymerase II and III transcription of snRNA genes. To establish this point, however, we need to purify the PSE-binding factor to homogeneity and test the pure preparation in RNA polymerase II and III functional assays. The pure preparation will also be used to obtain protein sequence and cDNA clones. We have purified the PSE-binding factor extensively and are now scaling up the preparation to obtain enough material for protein sequencing.

Characterization of the Inducer of Short Transcripts

M. Sheldon, F.C. Pessler, M.L. Sullivan, N. Hernandez

From our previous work, the IST was known to be located between positions −5 and +82 relative to the HIV-1 start site of transcription. Since most of this region is downstream from the start site of transcription, it was possible that IST was, like TAR, an RNA element. We have now completed the analysis of a large series of mutations encompassing the region from +1 to +82. These mutations define IST as a bipartite element, located at positions −5 to +26 and +40 to +59. The most important region for activity is that proximal to the promoter, but the +40 to +59 region is required for maximal activity. The RNA secondary structure is not important for IST function, and IST can be inverted without loss of activity, strongly suggesting that IST is a DNA element.

Some of the mutations we have analyzed debilitate IST but leave TAR intact. These constructs do not generate short transcripts and yet they are *trans*-activated by Tat. As a result, when transcription is measured close to the HIV-1 promoter such that both short and long transcripts are scored, the presence or absence of the IST element changes the apparent effect of Tat. With the IST intact, the HIV-1 promoter produces very few long transcripts and a large amount of short transcripts in the absence of Tat. In the presence of Tat, the number of long transcripts increases and the number of short transcripts is reduced concomitantly. Thus, the total number of transcripts derived from the HIV-1 promoter is unchanged in the presence and absence of Tat; only the ratio of these transcripts (long or short) is changed. Tat therefore seems to affect transcription elongation. However, when IST is debilitated, the HIV-1 promoter produces no short transcripts and still only few long transcripts in the absence of Tat. In the presence of Tat, the number of long transcripts is greatly increased. This time, the total number of transcripts is increased in the presence of Tat, and Tat therefore seems to affect transcription initiation. These results provide an explanation for the different effects of Tat observed by different groups in different systems. Most likely, IST is very active in systems (for example, transfected COS cells) in which Tat has been reported to act at the level of transcription elongation, and only slightly or not active in systems in which Tat has been reported to act at the level of initiation.

Identification of *trans*-Acting Factors Involved in IST Function

F.C. Pessler, M. Sheldon, M.L. Sullivan, N. Hernandez

In an attempt to identify the factor(s) involved in IST activity, we have screened fractions derived from HeLa cell nuclear extracts for the presence of factors that bind to probes containing an intact IST but not to probes containing a debilitated IST. We have identified an activity whose binding to IST and different IST mutations correlates well with IST activity. We are now in the process of characterizing and purifying this activity.

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TELOMERASE AND TELOMERE LENGTH REGULATION

C.W. Greider C. Autexier, A.A. Avilion, K. Collins, L.A. Harrington, L.L. Mantell, K.R. Prowse, S.K. Smith

Accurate chromosome transmission during cell division is essential to cell survival. When chromosome mechanics fail and whole chromosomes or parts of chromosomes are lost or rearranged, cell death or unregulated growth may occur. In contrast to broken chromosomes which undergo aberrant fusions and rearrangements, natural chromosome ends containing telomeres are stable. Telomeres are dynamic structures, shortening with chromosome replication and lengthening by the de novo addition of simple telomeric repeats. Telomere sequences are highly conserved in eukaryotes, consisting of short G-rich repeats, for example, TTGGGG in *Tetrahymena* and TTAGGG in humans and other mammals. Telomere G-rich repeats can be synthesized de novo onto chromosome ends by the enzyme telomerase, presumably to balance the inability of conventional DNA polymerases to replicate DNA ends completely. Telomerase is a ribonucleoprotein enzyme, in which the RNA component serves as an internal template for telomeric repeat synthesis. Telomerase, originally identified in *Tetrahymena*, has also been isolated from the ciliates, *Euplotes* and *Oxytricha*, as well as from immortalized human cell lines.

Recent experiments from our laboratory and others suggest that telomere length and telomerase activity may play a role in cellular senescence and immortalization. In primary cells, telomere length shortens with each round of cell division; thus, telomere length is an excellent biomarker for the replicative history of cells. Using this biomarker, we can follow the aging of tissues and primary cell cultures as well as the replicative history and thus the progression of some forms of cancer.

In the past year, we have greatly expanded our understanding of telomerase biochemistry. We identified and characterized a 3′ to 5′ nucleolytic cleavage activity in the *Tetrahymena* telomerase. This, along with the finding that short primers are elongated nonprocessively, led to a detailed model for telomerase primer elongation. We have also characterized both telomere shortening in vitro and tissue-specific developmental telomere length changes in the mouse *Mus spretus*. The results from these studies suggest that

the mouse is an excellent model organism in which to pursue the role of telomeres and telomerase in mammalian development and cancer. In characterizing the telomerase activity from mouse cells, we found that unlike its human counterpart, mouse telomerase is nonprocessive; it synthesizes only one repeat onto telomeric primers. Finally, the knowledge that some telomerase enzymes generate only short products led to the identification of telomerase in *Xenopus* oocyte extracts.

Purification and Reconstitution of Telomerase

L.A Harrington, K. Collins, C. Autexier

To study the telomerase enzyme mechanism in detail, our goal is to identify and clone the protein subunits and reconstitute activity from the purified components. To reach this goal, we have focused on identifying the polypeptide subunits that copurify with telomerase activity and on reconstituting activity with synthetic RNAs. Telomerase activity has been purified using a number of columns, including phenyl-Sepharose, DEAE, Heparin, Biorex, and sucrose gradients, to give an overall enrichment of 6000–10,000-fold. The proteins present in the highly purified fractions were visualized either by silver staining or by prelabeling the cells with ^{35}S followed by autoradiography. In the final step of purification using sucrose gradient sedimentation, we observed a separation of processive telomerase activity, which produces long product DNAs, from telomerase activity, which produces short addition products; the more processive enzyme sedimented farther into the gradient. Northern analysis showed that the telomerase RNA is present in fractions synthesizing both long and short products, although the peak cosediments with the short products. We are currently examining the basis for this distribution of processive and nonprocessive telomerase activities. Four prominent polypeptides reproducibly copurify with processive telomerase activity in the sucrose gradients. We are currently determining which of these polypeptides contribute to activity.

To understand the mechanism of telomerase fully, it will be necessary to reconstitute activity from purified components. We have used the purified fractions described above to begin reconstituting activity using synthetic RNA transcripts. Protein components were separated from the telomerase RNA using nuclease treatment or chromatographic separation of active telomerase fractions. A T7 transcript of the cloned RNA component was mixed with the protein fractions and assayed for telomerase activity under a variety of conditions, including different salt, pH, and $MgCl_2$ concentrations. Preliminary results suggest that some activity may be restored using the synthetic transcript. We are currently characterizing the activity in these reactions.

Primer Cleavage and Nonprocessive Elongation by *Tetrahymena* Telomerase

K. Collins

Our studies of *Tetrahymena* telomerase biochemistry led to the identification of two new activities: a 3′-5′ nucleolytic cleavage of primer or product DNA and a nonprocessive mode of elongation. We first noted a 3′-5′ nucleolytic cleavage activity in reactions with ^{32}P-labeled dGTP and dideoxythymidine triphosphate (ddTTP). Four primers with permuted telomeric repeat sequences ending in 0–3 dGTP residues, each 18 residues in length, were reacted with telomerase. The permuted primers $d(GGGGTT)_3$, $d(GGGTTG)_3$, $d(GGTTGG)_3$, and $d(GTTGGG)_3$ were elongated as expected from the sequence of the RNA template, except that the primer $d(GGGTTG)_3$ generated an extra labeled product at the size of the input, 18-residue primer (Fig. 1).

Several lines of evidence suggested that the primer-sized product of $d(GGGTTG)_3$ was generated by telomerase and not some unrelated DNA polymerase or nuclease activity. First, synthesis of both primer-sized product and other elongation products was sensitive to pretreatment of the extract with RNase. Second, the ratio of primer-sized to longer products remained constant in telomerase reactions across a glycerol gradient, indicating that the cleavage activity cosedimented with telomerase. Third, the K_m of primer activation for synthesis of the primer-sized product of $d(GGGTTG)_3$ was identical to the K_m of primer activation for the primer +1 nucleotide product of $d(GGGGTT)_3$. This would not be expected if a small fraction of the added primer was degraded by a contaminating exonuclease before

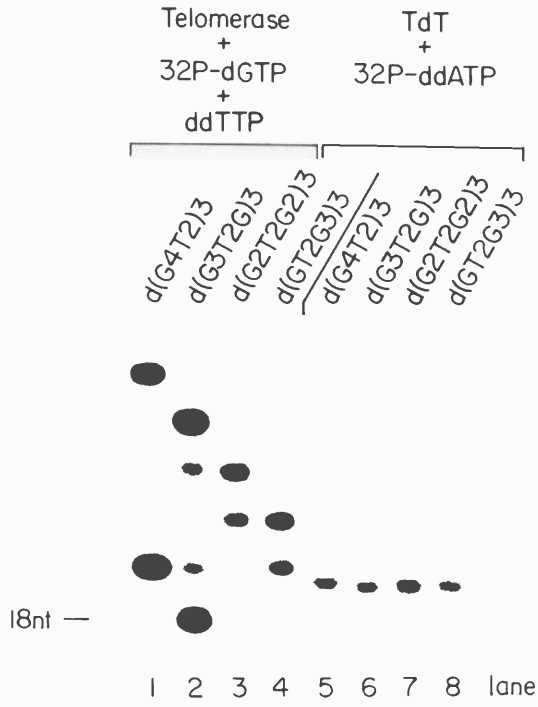

FIGURE 1 Cleavage of the primer $d(GGGTTG)_3$ by *Tetrahymena telomerase*. Telomerase reactions were carried out under standard conditions, and product DNA was precipitated, separated by PAGE, and subjected to autoradiography. Primers $d(GGGGTT)_3$ (lanes *1* and *5*), $d(GGGTTG)_3$ (lanes *2* and *6*), $d(GGTTGG)_3$ (lanes *3* and *7*), and $d(GTTGGG)_3$ (lanes *4* and *8*) were incubated with telomerase, ^{32}P-labeled dGTP, and ddTTP (lanes *1–4*). As a marker and control for the purity of the input primers, the same oligonucleotides were reacted with terminal deoxytransferase and ^{32}P-labeled ddATP (lanes *5–8*). The position of migration of an 18-residue telomeric primer is indicated at the left.

elongation by telomerase. Finally, using primers that after elongation are only very slowly released by telomerase, we showed that the cleavage reaction itself, separate from nucleotide addition, is sensitive to RNase treatment. These experiments suggest that telomerase catalyzes a template position-specific nucleolytic cleavage reaction. For the primer $d(GGGTTG)_3$, removal of the 3′deoxyguanosine was followed by addition of a ^{32}P-labeled dGTP nucleotide to generate a primer-sized, radioactively labeled $d(GGGTTG)_3$ product.

The primer specificity of the cleavage reaction suggested that cleavage occurred if primer or product sequence aligned with the cytosine residue at the extreme 5′end of the RNA template (3′-AACCCC AAC-5′), the last template position for nucleotide addition before repositioning of the product 3′end during processive elongation. We have extensively characterized the primer requirements for the cleavage reaction. Cleavage required a complete telomeric repeat with the permutation d(GGGTTG) at the primer or product 3′end. Cleavage was not detected for primers that have the correct 3′end permutation but lack a complete 3′telomeric repeat, such as $(GT)_2TTG$. When the primer $d(GGGTT G)_2GGGTTA$ was reacted with telomerase, the 3′dA was removed, and a ^{32}P-labeled dGTP was added in its place onto the 3′end. This and other experiments indicated that nucleotides other than deoxyguanosine could be removed from a primer 3′terminus, but only dGTP was initially added to the cleavage product. Finally, cleavage and subsequent nucleotide addition to the primer $d(GGGTTG)_2GGGTTTG$ demonstrated that more than one nucleotide could be removed by cleavage before elongation.

In addition to the cleavage reaction, we observed a second, novel property of telomerase: primer length, sequence, and concentration-dependent processivity. Previous studies using primers between 12 and 24 nucleotides in length showed that telomerase elongation is highly processive (Greider, *Mol. Cell. Biol. 11:* 4572 [1991]). Our more recent studies showed that at high concentration, primers less than ten residues in length efficiently stimulated nonprocessive synthesis by *Tetrahymena* telomerase. Using short primers with different permutations, we showed that these primers were "filled out" to the end of the 3′-AACCCCAAC-5′RNA template and then apparently dissociated from telomerase.

The identification of the cleavage reaction and nonprocessive elongation of short primers provide evidence for a revised model of telomerase action (Fig. 2). In this model, two sites are proposed to be involved in primer binding: the primer 3′end is bound at the "template site," 3′-AACCCCAAC-5′, whereas the adjacent primer sequence is bound at an "anchor site" (indicated by a shaded oval in Fig. 2). Processive synthesis requires that the product DNA remains bound to telomerase at the anchor site while dissociating from the template site with each round of 3′-end repositioning. The short primers elongated by telomerase in a nonprocessive manner may bind only at the template site, not at the anchor site, and thus be released from the complex when released from the template site.

After primer binding, elongation (added nucleotides shown in bold) results in extension of the primer 3′end toward the 5′end of the RNA template.

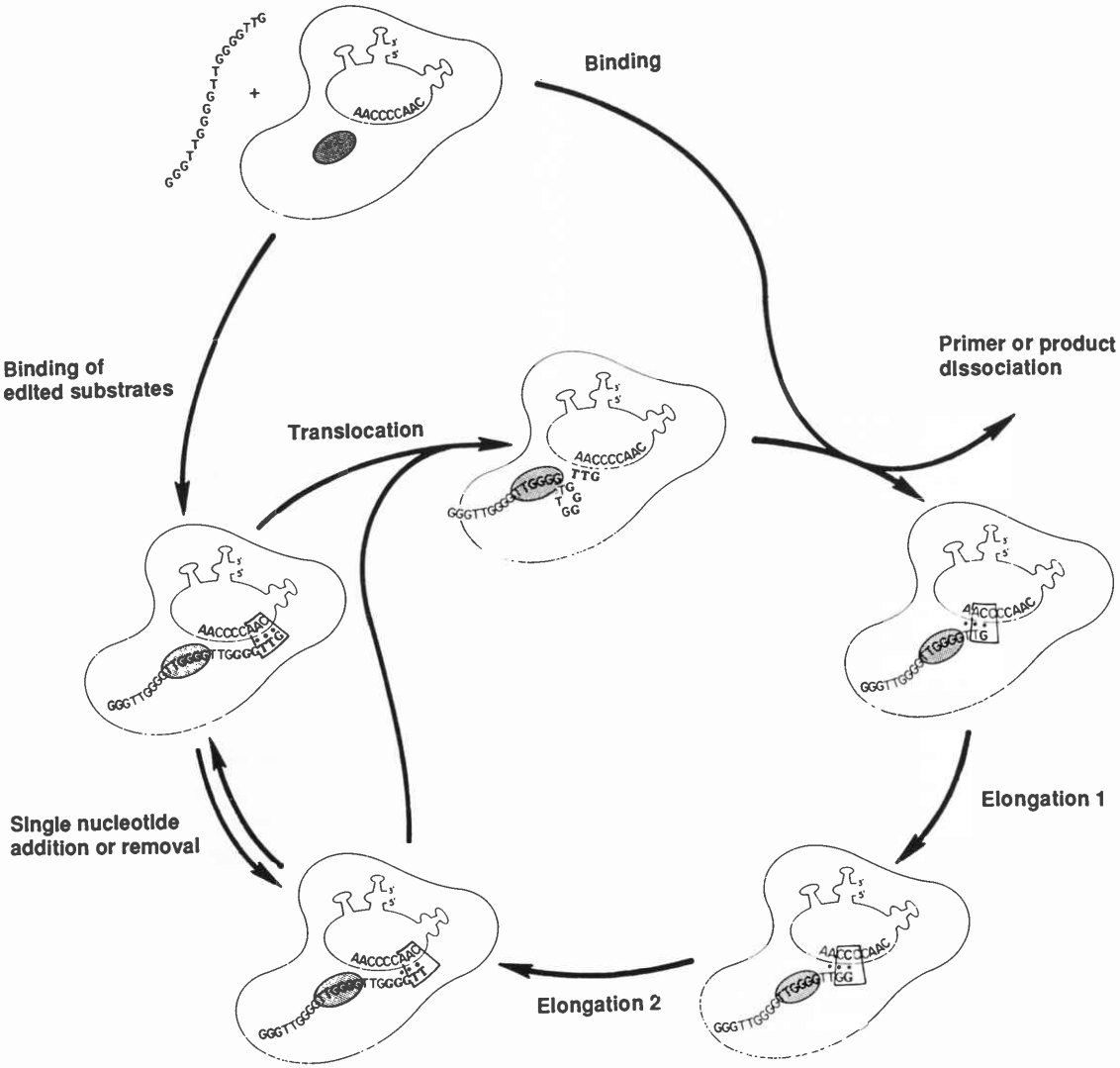

FIGURE 2 Model for product synthesis by *Tetrahymena telomerase*. Two sites are involved in the binding of primer; the 3′ end is bound at the template site, and the adjacent sequence is bound at the anchor site (*shaded oval*). Subsequent elongation steps, with nucleotides added shown in bold, result in elongation of the primer 3′ end toward the the 5′ end of the RNA template. Translocation or repositioning of the product 3′ end to the 3′ end of the template occurs after copying of the template sequence. The 3′ end of the primer or elongated product bound at the 5′ end of the template sequence can also undergo cleavage. Base-pairing interactions between the template RNA and primer or product DNA are indicated by dots between base-paired residues.

Repositioning of the product 3′ end to the 3′ end of the template site, termed translocation, can occur after complete or almost complete copying of the template sequence. Repositioning may occur either before or after addition of the dGTP encoded by the most 5′ template cytosine residue. Instead of repositioning, the 3′ end of primer or product bound at the 5′ end of the template can also undergo nucleotide cleavage. The cycle begins again when the product 3′ end reestablishes stable alignment with the 3′ end of the template. In testing the predictions of this new model, we will be able to further define the novel mechanism of DNA synthesis carried out by the telomerase enzyme.

Mouse Telomere Shortening In Vitro and In Vivo

K.R. Prowse

Our recent experiments (Counter et al. *EMBO J. 11:* 1921 [1992]) showed that telomerase activity was not detectable in cultures of primary human cells when telomeres were shortening. However, immortalized clones arising from SV40 T-antigen-transformed primary cells had both stable telomere length and detectable telomerase activity. This suggests that activation of telomerase may be essential for the growth of immortalized human cells. To further explore the role of telomerase in cellular senescence and immortalization, we have extended our studies of telomere length and telomerase activity to mice.

Primary mouse fibroblast cells have been well studied and characterized in vitro. A fundamental difference exists between mouse and human cells in culture: Human fibroblasts rarely, if ever, spontaneously immortalize, whereas mouse fibroblasts in culture spontaneously immortalize at a high frequency. Studies of telomere length and telomerase activity during immortalization of both human and mouse cells may add to our understanding of these differences and their implications for cancer.

Mouse telomeres contain the same $(TTAGGG)_n$ repeated sequence found in humans, although the lengths of the terminal restriction fragments in *Mus musculus* and *Mus domesticus*, ranging from 50 to 150 kbp, are significantly longer than in humans. Therefore, if a decrease in telomere length similar to that seen in human cells occurred with increasing replicative age, it would be extremely difficult to detect in these mice. However, *Mus spretus*, a wild-derived mouse species, possess terminal restriction fragments of only 5 to 15 kbp in length, similar to humans, and thus any changes in telomere length should be detectable. For this reason, *M. spretus* cell cultures and tissues were used to examine telomere length changes both in vitro and in vivo.

Primary *M. spretus* fibroblasts were obtained from newborn mouse skin and were grown in culture. The growth kinetics and morphology of the cells were monitored to determine when the population underwent crisis and spontaneously transformed. At various times during the cell growth, genomic DNA was isolated and digested with restriction enzymes, and telomere length was analyzed by hybridization to the telomeric oligonucleotide $d(TTAGGG)_3$ (Fig. 3). In a separate experiment, a *Bal*31 exonuclease digestion showed that most of the $d(TTAGGG)_3$ hybridizing bands were truly telomeric. During growth of the cells in culture, the terminal restriction fragments decreased in length with increasing mean population doubling (MPD). After the culture had passed through crisis, the length of the terminal restriction fragments remained stable. These results are similar to what we found using human cells in culture.

The *M. spretus* fibroblasts were also examined for telomerase activity both before and after crisis. S100 cell extracts were prepared, fractionated over DEAE columns, and assayed for telomerase activity. The results showed that although no telomerase activity was detected before crisis, telomerase activity was detected after the culture had undergone crisis and the cells were immortalized. The lack of detectable telomerase activity when telomere lengths are shortening, coupled with the detection of activity after crisis when telomere lengths are stable, suggests that telomerase activity is required to maintain telomere length in transformed cells.

The finding that telomeres shorten during growth of mouse cells in culture allows us to use the mouse as a model system to study telomere length during aging in vivo. We found that telomere lengths in tissues from individual newborns were similar, whereas telomere lengths from tissues of individual adults differed. The most striking difference was observed in the length of testes telomeres. Testes telomere length was significantly longer in adult mice than in other tissues. This suggests that a developmentally regulated telomere length increase may occur after birth in the mouse. To test this hypothesis, we studied both telomere length and telomerase activity at various stages of mouse development. At approximately 5 weeks after birth, the length of testes telomeres increased relative to other tissues from the same mouse. This increase in telomere length coincides with the appearance of the first mature sperm cells in the male mouse. Telomerase activity was assayed in testes from mice 2, 4, 6, 8, 10, 13, and 16 weeks of age. Activity was not detected in 2-week-old mice but was found in those mice 4 weeks and older, which suggested that telomerase activity may be regulated during spermatocyte development. To determine whether telomerase activity came from the germ-line cells in the testes, testes from mutant W/W(V) mice, which have a complete lack of primary spermatogonia, were assayed for telomerase activity. No activity was detected in 9-week-old W/W(V) mice,

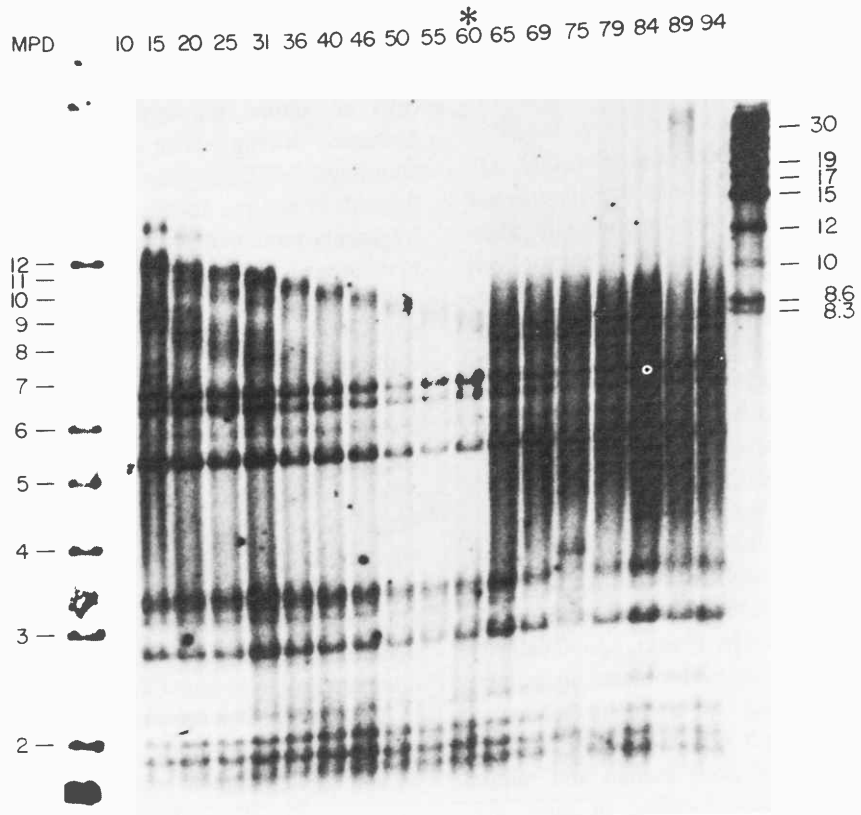

FIGURE 3 Telomere shortening in mouse fibroblasts. Genomic DNA from *M. spretus* fibroblasts was isolated at the indicated mean population doubling (MPD), and 2-μg samples were digested with *Hin*fI and separated in a 0.5% agarose gel. The gel was dried, denatured, neutralized, and hybridized to a ^{32}P-labeled $(TTAGGG)_3$ oligonucleotide. The asterisk indicates the MPD at which the population of cells was in crisis. The numbers at the left and right indicate the molecular weights of the marker DNA.

whereas normal age-matched siblings did have telomerase activity.

These studies in mice support and extend our previous studies on telomere length and telomerase regulation in human cells during both cellular senescence and immortalization. The observation that mouse telomere length regulation is similar to that of humans allows the use of mice to dissect telomere regulation in vivo. To directly test the role of telomerase in aging and immortalization, we will need to alter telomerase and determine the affect on both cell viability and organismal development. A thorough characterization of mouse telomere regulation will provide the background for future studies using transgenic mice with artificially regulated telomerase.

Mouse Telomerase Generates Only Short Addition Products

K.R. Prowse

In the course of our studies on mice, we found that mouse telomerase has biochemical properties very different from telomerases characterized from both ciliates and human cells. Telomerase activity was identified in extracts from several different mouse cell lines. Addition of telomeric $d(TTAGGG)_n$ repeats was specific to telomeric oligonucleotide primers and sensitive to pretreatment with RNase A. In contrast to the hundreds of repeats synthesized by the human and *Tetrahymena* telomerase enzymes in vitro, mouse telomerase synthesized only one or two

d(TTAGGG)$_n$ repeats onto telomeric primers. The products observed after elongation of primers with circularly permuted d(TTAGGG)$_3$ sequences and after chain termination with ddATP or ddTTP indicated mouse telomerase pauses after the addition of the first dG residue in the sequence TTA*G*GG (Fig. 4). The short length of the products synthesized by mouse telomerase was not due to a diffusible inhibitor in the mouse extract, because the human telomerase continued to synthesize long products when mixed with mouse fractions. Primer challenge experiments showed that the human enzyme synthesized long d(TTAGGG)$_n$ repeats processively in vitro, whereas the mouse telomerase was much less processive. The identification of short telomerase reaction products in mouse extracts suggests that extracts from other organisms may also generate only short products. This knowledge may aid in the identification of telomerase activity in organisms where activity has not yet been detected.

Identification of Telomerase in *Xenopus* Oocytes

L.L. Mantell

As described above, we found that telomerase activity was present in mouse testes extracts. This sup-

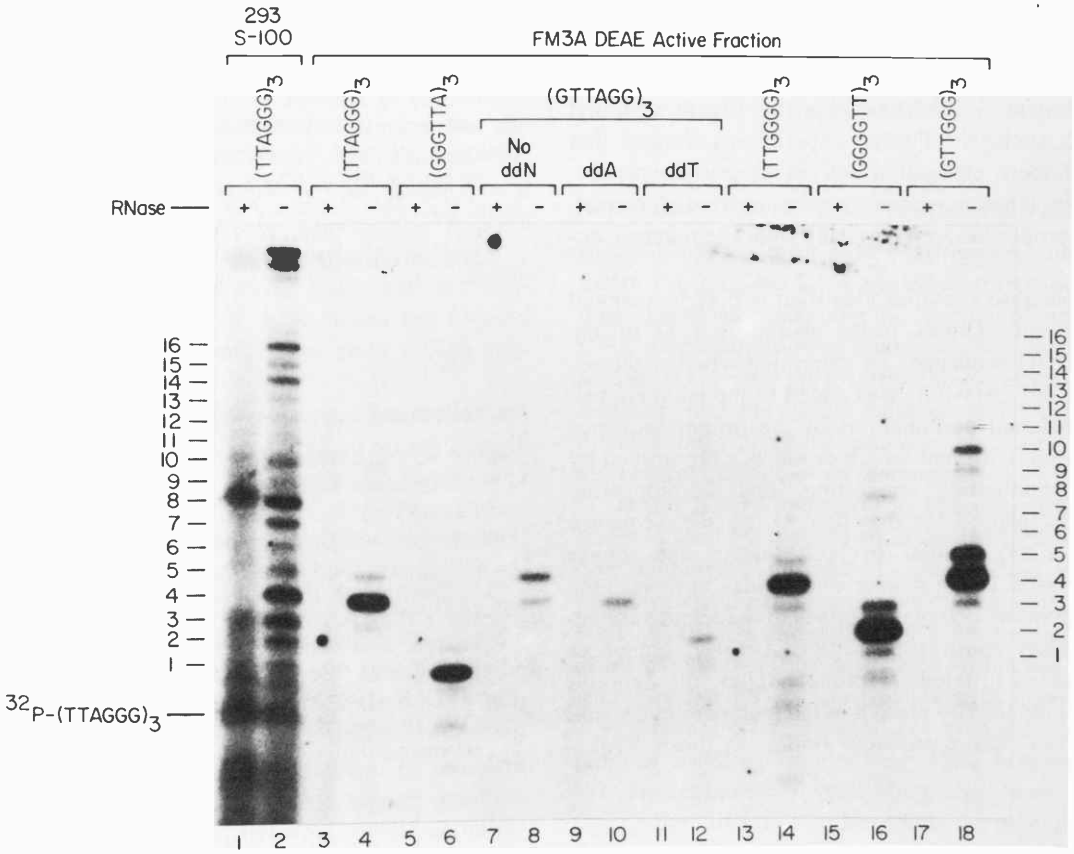

FIGURE 4 Mouse telomerase synthesizes short products. An FM3A DEAE fraction was assayed for telomerase activity using different telomeric oligonucleotides as primers. (Lanes *1,2*) 293 S100 extract, (TTAGGG)$_3$; (*3–18*) FM3A DEAE fraction; (*3,4*) (TTAGGG)$_3$; (*5,6*) (GGGTTA)$_3$; (*7–12*) (GTTAGG)$_3$; (*13,14*) (TTGGGG)$_3$; (*15,16*) (GGGGTT)$_3$; (*17,18*) (GTTGGG)$_3$. (Lanes *9,10*) 0.5 mM ddATP was used instead of dATP; (lanes *11,12*) 0.5 mM ddTTP was used instead of TTP. The position of a ^{32}P-labeled (TTAGGG)$_3$ is indicated. The numbers at the left and right indicate the position of the additional nucleotides added to the input,18-residue primer. +/– indicates plus or minus RNase pretreatment.

ports our model that telomerase activity in germ cells allows maintenance of telomere length in these cells, although in some somatic tissues telomeres shorten with age. To extend our studies of germ-line cells, we wanted to study telomerase in oocytes. We chose to analyze oocytes from the frog *Xenopus laevis*.

Although *Xenopus* telomeres have not been cloned and sequenced, in situ hybridization and Southern blots suggest that *Xenopus* has the same telomere sequences, $d(TTAGGG)_n$, as found in other vertebrates. Thus, *Xenopus* telomerase is expected to synthesize $d(TTAGGG)_n$ sequences onto telomeric DNA primers. Stage VI oocytes were dissected from adult *Xenopus* ovaries and treated with progesterone to allow maturation. S100 extracts made from these matured oocytes were fractionated on a DEAE column and telomerase assays were carried out.

No telomerase activity was detected in S100 extracts; however, the 0.2 M NaCl fraction from a DEAE column contained a product band that closely resembled that synthesized by mouse telomerase. Pretreatment of the extract with RNase inhibited product synthesis. Further experiments showed that this telomere elongation activity is primer-specific: Only the telomere sequence primers were elongated. These properties are consistent with a telomerase activity.

Telomerase activities identified in both human and mouse cell extracts pause at the first G of the dTTA*G*GG sequence. To determine whether the sequence $d(TTAGGG)_3$ was added to the telomere primer, different permutations of the primer sequence were used. Different length products were primed by each permutation, suggesting that the elongation products paused at the first G of the sequence d(TTA*G*GG), similar to both human and mouse telomerases. Using dideoxynucleotides to replace the corresponding deoxynucleotides in the telomerase activity assay confirmed that $d(TTAGGG)_n$ sequences were added to telomere primers. Thus, the *Xenopus* telomerase activity present in the mature oocytes generates only short products similar to those synthesized by mouse telomerase. Both cell cycle and developmental regulation are well characterized in *Xenopus*. The identification of telomerase in oocytes provides the opportunity to use *Xenopus* to explore telomerase regulation further.

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MOLECULAR CHROMOSOME STUDIES OF *ARABIDOPSIS THALIANA*

E.J. Richards T. Kakutani A. Vongs
A. Kelly M. Walsh

We are studying the organization of higher eukaryotic chromosomes using the flowering plant, *Arabidopsis thaliana*, as a model system. Our research applies both genetic and molecular biology approaches. During the past year, we focused on two projects: (1) study of telomeric DNA structure and (2) characterization of DNA methylation mutants.

CHARACTERIZATION OF TELOMERIC DNA FROM *A. THALIANA*

Telomeres are unusual chromatin domains that cap the termini of linear eukaryotic chromosomes. The DNA sequences at the extreme termini are composed of tandem arrays of short, G-rich repeats. We have isolated several *A. thaliana* telomeric DNA clones by functional complementation in yeast, and our continuing characterization of these clones has focused this past year on telomere repeat array (TRA) substructure.

We have determined the primary nucleotide sequence of three isolated TRAs, all of which contain repeats that vary from the canonical *A. thaliana* telomere repeat, 5′-TTTAGGG-3′. The variant repeats are clustered in the centromere-proximal region of the TRA. These variants tend to be uniform in size and sequence and generally deviate from the canonical repeat at only one position. At the centromere-proximal boundary of the TRA, the variant repeats give way to degenerate repeats that vary widely in size and sequence. In two of the sequenced TRAs, the simple gradient of uniform (TTTAGGG)→variant→degenerate repeats→centromere is replaced by a patchwork of subdomains. Most striking is the substructure of one TRA from clone YpAtT5 that contains a block of nontelomeric sequence roughly 100 bp in length within the TRA. We have postulated that the patchwork substructure arises from unequal crossing over in the TRAs or by small translocations between TRAs.

Although it is generally assumed that all telomeres within a cell are the same, we have shown that telomere repeat variants can be chromosome- and strain-specific. One of the common telomere repeat variants we found, TTCAGGG, is present in Landsberg strains of *A. thaliana* but is absent in the common laboratory strain, Columbia. Moreover, TTCAGGG repeats are only detectable on a subset of Landsberg telomeres.

The presence of chromosome- and strain-specific telomere repeats illustrates the fluidity of the genome at chromosome termini. We hope to study this fluidity further by following the telomere structural polymorphisms we have identified.

A. THALIANA DNA HYPOMETHYLATION MUTANTS

In collaboration with Rob Martienssen, our group isolated *A. thaliana* DNA methylation mutants to address questions concerning the function of eukaryotic DNA modification using genetic tools. Southern blot screens were used to identify three independent *A. thaliana* mutants with reduced levels of DNA methylation. During the past year, we have extended our characterization of these mutants.

Despite a 70% reduction in cytosine methylation levels caused by the two most severe hypomethylation mutations, designated *ddm1-1* and *ddm1-2*, homozygous mutant plants develop normally and exhibit no striking morphological abnormalities. However, the mutations are associated with a segregation distortion phenotype since plants with hypomethylated genomes are significantly overrepresented in the progeny resulting from selfing *ddm1* heterozygotes. We are currently investigating the cause of the segregation distortion to learn how the plant cell uses DNA modification.

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STRUCTURE AND COMPUTATION

This section consists of five laboratories interested in computational biology and the detailed structural properties of proteins. Dr. John Anderson is a macromolecular crystallographer studying both the endonuclease and methyltransferase of the *Pvu*II restriction-modification system. A crystal structure for a complex between the *Pvu*II restriction enzyme complexed with DNA is now close at hand. Dr. Jeff Kuret's laboratory studies protein kinases including casein kinase 1 and the cAMP-dependent protein kinase. Dr. Tom Marr is a computational biologist whose laboratory is heavily involved in developing software to aid in the assembly of genomic mapping and sequencing information as well as its analysis. Dr. Jim Pflugrath is a crystallographer working on the neurotrophic factor S100β. He also studies the yeast cAMP-dependent protein kinase in collaboration with Dr. Jeff Kuret, and a refined model for this protein has recently been achieved. Dr. Pflugrath has also been instrumental in developing software to aid in the collection of X-ray data using area detectors. Dr. Rich Roberts' laboratory has had a long-standing interest in restriction enzymes and methylases. Most recently, in collaboration with Drs. Pflugrath and Cheng, the first high-resolution structure has been obtained for a DNA cytosine methyltransferase. The newest member of this section is Dr. Xiaodong Cheng, who originally joined Cold Spring Laboratory as a postdoctoral fellow with Dr. Jim Pflugrath and who has now established his own independent laboratory.

During the past year, Dr. R.J. Roberts has moved from Cold Spring Harbor Laboratory to join New England Bio-Labs, from where he will continue an active collaboration with Dr. Xiaodong Cheng.

MACROMOLECULAR CRYSTALLOGRAPHY

J.E. Anderson
J.W. Pflugrath
G.K. Balendiran
K. Bilchick
D. Cahill
C.K. Cheung
J.R. Horton
J. Keller
T. Malone

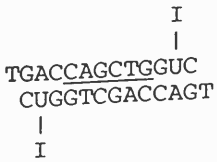
*Pvu*II Endonuclease and DNA

G.K. Balendiran, J. Keller, J.E. Anderson [in collaboration with I. Schildkraut, New England Bio-Labs, and R.M. Blumenthal, Medical College of Ohio]

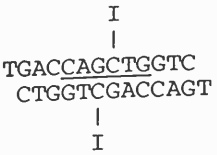
The restriction endonuclease from *Proteus vulgaris*, R·*Pvu*II, recognizes the DNA sequence CAGCTG. In the presence of magnesium, R·*Pvu*II cleaves between the central guanine and cytosine bases. We have cocrystallized R·*Pvu*II with the following oligodeoxynucleotide carrying a *Pvu*II recognition sequence (underlined):

TGACCAGCTGGTC
CTGGTCGACCAGT

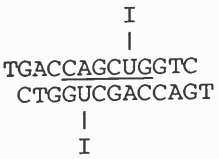
To prevent cleavage of the oligo, EDTA is included in the crystallization buffer. The crystals are space group $P2_12_12_1$ with unit cell dimensions a = 95.1 Å, b = 85.6 Å, c = 47.7 Å, and diffract to at least 2.6 Å resolution. We have collected native data to 3 Å resolution. To obtain phases to calculate an electron density map, we have crystallized R·*Pvu*II with the following derivative oligos, which have 5-iododeoxyuracil or 5-iododeoxycytosine at the indicated positions:



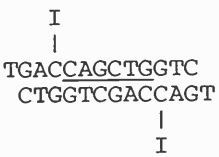
derivative 1



derivative 2



derivative 3



derivative 4

Complexes between R·*Pvu*II and derivative oligos 1, 2, and 3 crystallize isomorphously and so could provide phases for the native data. The iodine positions for derivative 1 were determined, and a single isomorphous replacement (SIR) map was calculated, but the map was uninterpretable. Data have been collected for derivatives 2 and 3, and processing is under way. Once phases are determined, we will calculate a multiple isomorphous replacement (MIR) electron density map and begin building a model of the complex. R·*Pvu*II crystallizes with derivative oligo 4, but the crystals are not isomorphous.

*Pvu*II Methylase

C.K. Cheung, J. Keller, K. Bilchick, J.E. Anderson
[in collaboration with R.M. Blumenthal, Medical College of Ohio, and I. Schildkraut, New England Bio-Labs]

We have purified M·*Pvu*II, which methylates the N4 atom of the cytosine in the fourth position of the *Pvu*II recognition sequence. A variety of proteases cleave M·*Pvu*II into two to four relatively stable fragments. The pattern of fragments produced by protease K depends on the presence or absence of the essential cofactor *S*-adenosylmethionine (AdoMet), suggesting that AdoMet affects the conformation of the methylase. Intact M·*Pvu*II has so far been resistant to crystallization. If it continues to be so, proteolytic fragments of M·*Pvu*II will be purified, since they may crystallize more readily.

Stichodactyla helianthus Cytolysin III

J. Keller, J.E. Anderson [in collaboration with W. Kem, University of Florida College of Medicine]

Cytolysin III (cIII) is a 153-residue protein that is found in the skin of the Caribbean sea anemone *Stichodactyla helianthus*, possibly associated with nematocysts, the stinging cells of the anemone. It probably plays both offensive and defensive roles and appears to function by oligomerizing and forming pores in cell membranes, leading to osmotic lysis of target cells. In view of its function as an integral membrane protein, we were surprised when we were able to concentrate purified cIII to over 100 mg/ml! However, it is probably stored at very high concentrations in the skin of the anemone before being released and inserting into target cell membranes, and this may explain the unusually high aqueous solubility of cIII. We have obtained small crystals of cIII under several conditions. One of these includes a high detergent concentration, which might be expected of a membrane protein. The crystals are too small for diffraction analysis, and we are attempting to increase their size.

Structural Studies of the Neurotrophic Factor S100β

D. Cahill, T. Malone, J.W. Pflugrath [in collaboration with D. Marshak, Cold Spring Harbor Laboratory]

S100β is a member of a family of dimeric acidic Ca^{++}-binding proteins involved in cell differentiation, cell cycle progression, and calcium signal transduction. S100β is synthesized and released from astrocytes in the brain, and its disulfide-linked dimeric form has neurotrophic activity on cerebral cortical neurons in vitro. Elevated levels of S100β protein, mRNA, and specific neurotrophic activity in autopsy samples of Alzheimer's disease patients sug-

gest the possibility that S100β contributes to Alzheimer's disease neuropathology. S100β is homologous to proteins with EF-hand calcium-binding motifs, such as calmodulin and intestinal calcium-binding protein (ICaBP). We are attempting to determine the crystal structures of S100β at pH 7 and pH 8 from existing crystals of S100β that diffract beyond 1.6 Å resolution. We have grown at least three different crystal forms of S100β that may reveal for the first time conformational differences in a single member of the Ca^{++}-modulated protein family. The conformation of the extended amino and carboxyl termini of S100β and the quaternary structure of a S100β protein will be seen for the first time. The nature of any change in dimer interaction needed to form intermolecular disulfide bonds and how calcium binding either prevents or enhances any change also will be unveiled. Finally, the neurotrophic activity of S100β, its role in the development and maintenance of neuronal function and in Alzheimer's disease, and its similarity to many other proteins involved in growth regulation, cell cycle progression, and cystic fibrosis suggest the possibility of the development of new diagnostic and therapeutic agents based on the S100β structure.

S100β has significant sequence homology with ICaBP, whose structure has been refined to high resolution; thus, we have used molecular replacement techniques to try to solve for the phases of the previously collected S100β native data. We aligned the 92 residues of recombinant rat S100β with the 75 residues of ICaBP on the basis of amino acid identity, surface accessibility, and secondary structure elements. The following are the main features of the alignment: (1) Nearly every residue of ICaBP is either identical with (28 of 75) or similar to (21 of 75) its corresponding residue of S100β unless it is on the protein surface; (2) S100β has amino- and carboxy-terminal extensions when compared to ICaBP; (3) only a single three-residue hydrophilic insertion at position 45 (a surface loop of ICaBP) is needed for the alignment; (4) S100β Cys-68 is aligned with ICaBP Val-61, which is completely buried in the calcium-bound form of EF hands; and (5) Cys-84 is in the carboxy-terminal extension of S100β and has no counterpart in ICaBP. This alignment predicts calcium cannot bind to the second EF hand if Cys-68 is part of a disulfide bond linkage as is expected to occur in a S100β covalent dimer.

The rotation search was done first using the ICaBP structure, then the ICaBP C_α skeleton with the S100β sequence-aligned side-chain replacements, then the separated EF hands of this model individually, and finally the side-chain model with residues 37–43 deleted. Several resolution ranges were tried with three different software packages. The rotation function results were used in translation searches. The packing of models from the best solutions in the S100β unit cell was also checked. Finally, some of the solutions were subjected to simulated annealing with restrained least-squares refinement. Refinement never proceeded to a low enough R-factor to lead us to believe that any of our molecular replacement solutions were correct. We are now pursuing isomorphous replacement as a method of solving the phase problem for our S100β crystals.

Yeast cAMP-dependent Protein Kinase

J.W. Pflugrath, T. Malone, J. Kuret

Protein kinases are important and conserved components of many regulatory pathways because they can integrate several input signals and coordinate a response through the phosphorylation of a limited number of substrate proteins. The three-dimensional crystal structure of the cAMP-dependent protein kinase will allow us to define the structural basis of enzyme/substrate recognition and to assess the implications of this information for the protein kinase family in general.

We have crystallized the cAMP-dependent protein kinase catalytic subunit from *Saccharomyces cerevisiae*. The kinase catalytic subunit crystallizes in space group $P6_522$ with cell dimensions $a = b = 61$ Å, $c = 320$ Å. We have grown crystals as large as 0.6 by 0.6 by 1.5 mm that have diffracted beyond 2.7 Å resolution with synchrotron radiation. Data from native and pCMBS-soaked crystals were recollected at beamline X12C of the National Synchrotron Light Source at Brookhaven National Laboratory. A new version of the area detector software MADNES, which allows one to simultaneously display the raw images and predicted reflections, revealed discrepancies in the indexing of some crystals. When this was corrected, the R_{merge}s for both the native and the derivative data sets were 8%.

Single isomorphous replacement refinement with anomalous scattering yielded phases with a figure of

merit of 0.70, and these were used to calculate an electron density map that was interpretable. This new map confirmed our previous preliminary partial sequence fit from earlier maps and allowed us to visually rotate the mouse cAMP-dependent protein kinase structure (Knighton et al., *Science 253:* 407 [1991]) onto our density map. It was immediately apparent that the amino- and carboxy-terminal domains were oriented differently with respect to each other in the yeast and the mouse kinase structures. The mouse structure had been solved with a bound protein kinase inhibitor peptide, whereas our yeast kinase was an apo structure without any ligands present in the crystals. After model building and restrained least-squares refinement, the crystallographic R-factor is 22% for 8210 reflections from 20 to 2.8 Å resolution. The model stereochemistry was restrained throughout refinement with the final rms deviations from ideality of 0.015 Å for bonds, 2.5° for bond angles, and 0.020 Å for planes. Figure 1 shows a diagram of the final refined model. We are in the process of analyzing the differences between the unbound protein and the protein substrate complexes.

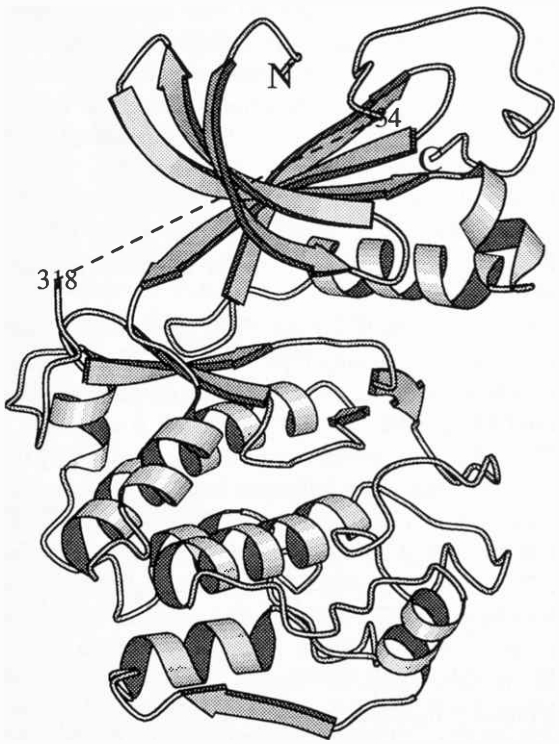


FIGURE 1 Topological diagram of the structure of cAMP-dependent protein kinase catalytic subunit (TPK1Δ) from *S. cerevisiae*. Shown are residues 38 to 318 and residues 334 to 350. Electron density for residues 36–37 and 318–334 is not seen in the Fourier maps.

Cell Cycle Protein Regulators

J.R. Horton, J.W. Pflugrath

We have initiated a project to determine the three-dimensional crystal structures of the major macromolecular switches of the cell cycle. These switches are complexes that contain at least two proteins: a cdc2-like protein (the catalytic component) and a cyclin (the regulatory component). These switches are strongly controlled by other proteins that can turn them "on" or "off." In the activated "on" state, these protein complexes have serine/threonine kinase activity and phosphorylate other proteins to bring about the major events of the cell cycle.

Cyclin D1 is a 36-kD protein involved in regulating some events near the G_1/S transition of the cell cycle. Overamplification of the cyclin D1 gene has been linked to the development of several types of human cancer. An *Escherichia coli* strain containing the gene for cyclin D1 has been obtained from Drs. Xiong and Beach here at Cold Spring Harbor Laboratory. Unfortunately, although a large amount of protein is expressed (40–50 mg per liter cell culture), all of this protein is found in inclusion bodies. Presently, we are purifying this protein and hope to crystallize it soon.

Area Detector Software

J.W. Pflugrath [in collaboration E.M. Westbrook, Argonne National Laboratory]

To elucidate macromolecular structures, crystallographers must measure thousands of diffracted intensities from numerous crystals. Very often, crystals are too small, diffract too weakly, have large unit cells, or are very sensitive to radiation damage, so that usable data are unobtainable with a conventional laboratory X-ray source. However, useful data from such crystals may be measured with a synchrotron source that offers X-rays 100–1000 times brighter, no beam divergence, and a tunable wavelength. To maximize the usefulness of synchrotron radiation, state-of-the-art area detectors are being developed with high count rates, high dynamic range, low noise, fast readout, and a large area coverage. One such detector is the 1024 by 1024 pixel charge-coupled device (CCD) area detector developed at Argonne National Laboratory and installed at beamline X8C of the Na-

tional Synchrotron Light Source. This beamline and detector are part of a National Institutes of Health Research Resource that macromolecular crystallographers can use for crystal structure determinations. We are responsible for all of the application software at this resource.

In the past year, we have written, installed, and tested a set of device handlers for the major hardware components (the detector, the goniometer, the ion chamber counters, and the shutter) for the X8C research resource. These are used in a new version of MADNES, the device-independent area detector software developed principally by us. This version directly controls the detector hardware that simplifies the user interface, reduces training time, and results in fewer errors in data collection. Other enhancements to MADNES are visualization tools that display images either singly or overlayed (contributed by Phil Evans, MRC) and a graphical user interface (contributed by John Skinner and Robert Sweet, BNL). Although MADNES cannot make the crystals diffract better, it can help to maximize any results obtained with the state-of-the-art CCD detector. This software has also been exported to several other laboratories that are designing and building their own detectors.

PUBLICATIONS

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Pflugrath, J.W. 1992. Developments in X-ray detectors. *Curr. Opin. Struct. Biol.* **2:** 811–815.

MACROMOLECULAR CRYSTALLOGRAPHY

X. Cheng K. McCloy

Crystal Structure of *Hha*I DNA Methyltransferase Complexed with *S*-adenosyl-L-methionine

X. Cheng [in collaboration with S. Kumar and R.J. Roberts, Cold Spring Harbor Laboratory]

The three-dimensional structure of the DNA (cytosine-5)-methyltransferase, M·*Hha*I, complexed with *S*-adenosyl-L-methionine (AdoMet) has been determined at 2.5 Å resolution. The molecule comprises two domains. The large domain consists of a large α/β domain containing most of the conserved sequences among all DNA (cytosine-5)-methyltransferases and is responsible for the binding of cofactor AdoMet and providing catalytic nucleophile cysteine 81. The small domain of predominantly β strands contains the "variable region" responsible for sequence specificity. A cleft between the two domains is likely to contain the active site of the molecule, where the binding of AdoMet and DNA can occur in close proximity to the catalytic center.

DNA methyltransferases are found in many organisms ranging from bacteriophages to mammals. They recognize specific DNA sequences and transfer a methyl group from the cofactor AdoMet to adenine or cytosine residues. In prokaryotes, DNA methylation is used by the restriction-modification system to distinguish between host and foreign DNA. In eukaryotes, DNA methylation has been implicated in the control of gene expression, epigenesis, and genomic imprinting. Recently, the mouse methyltransferase was found to be associated with the DNA replication center in a cell-cycle-dependent manner and was shown to be essential for embryonic development.

M·*Hha*I from the bacterium *Haemophilus haemolyticus* methylates the internal cytosine of its recognition sequence 5′-GCGC-3′. It contains 327 amino acids with a molecular weight of 37,000. The structure of *Hha*I methyltransferases complexed with the methyl donor, AdoMet, was not only the first to be solved for any DNA methyltransferase, but also the first for any methyltransferase that utilizes the ubiquitous methyl donor AdoMet. Due to the conserved nature of (cytosine-5)-methyltransferases, the information obtained from this structure can be generalized to the entire family, including the mammalian CpG

methyltransferase. This structure will assist in elucidating the mechanisms of the methylation process, including substrate binding and activation, nucleophilic catalysis, and cofactor binding. It will also aid in understanding DNA binding and sequence discrimination by catalytic protein.

STRUCTURE DETERMINATION

Crystals of *Hha*I methyltransferase complexed with the cofactor AdoMet were monoclinic space group $P2_1$ with two molecules per asymmetric unit and had unit-cell dimensions of a = 55.3 Å, b = 72.7 Å, c = 91.0 Å, and β = 102.5° (Fig. 1). The structure was solved with the phases derived from two mercurial derivatives (Fig. 2). The initial 3 Å resolution MIRAS (multiple isomorphous replacement with anomalous scattering) map was of sufficient quality to place α-helixes and β-strands in recognizable densities, and a partial, discontinuous polyalanine model was fitted to the electron density. The two molecules forming the crystallographic asymmetric unit are related by a noncrystallographic twofold axis of rotation. Two AdoMet molecules were also clearly identified in the MIRAS map. Phases were further improved by averaging about the twofold noncrystallographic axis and solvent flattening. Three rounds of model building, averaging with the MIRAS phases, allowed an unambiguous trace of the known amino acid sequence to the electron density map. After the initial model building, the twofold averaging was discontinued, and the atomic model was subjected to least-squares refinement with both monomers treated independently. The current model consists of all residues (aa 1:327) and AdoMet for both monomers. No water molecules or sulfate anions have been included in the model. The current R-factor is 0.227 for 20,057 reflections between 8- and 2.5 Å resolution.

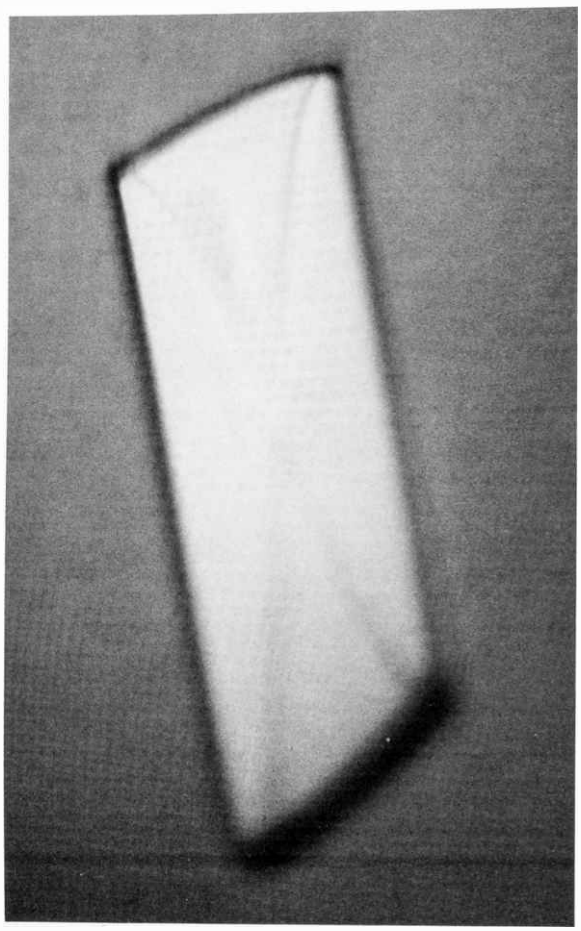

FIGURE 1 Photomicrograph of the crystallized M·*Hha*I-AdoMet complex grown by the hanging drop vapor diffusion method at room temperature. The crystal shown has dimensions 0.9 mm x 0.3 mm x 0.3 mm.

TOPOLOGICAL OVERVIEW

The structure of M·*Hha*I methyltransferase is illustrated schematically in Figure 3. The molecule of size 40 x 50 x 60 Å is folded into two domains, a large domain (aa 1–193 and aa 304–327) and a small domain (aa 194–275), with a hinge region (aa 276–303) connecting them. The large domain is a mixed α/β structure consisting of the amino-terminal two thirds of the protein followed by a crossover connection to an additional helix (αG) from the carboxyl terminus. There are two β-α-β structural elements and one hairpin element. The twisted six-strand β sheet contains four adjacent parallel β strands, numbered 4, 3, 1, 2, whereas strands 5 and 6 form a β hairpin next to strand 4. The mixed β sheet is in the middle and sandwiched by two layers of structures: helices αC and αD above and αA, αG, and strand β7 underneath. Helix αB runs across the sheet in front of the sandwich. The small domain consists of seven strands. The five up-and-down antiparallel strands (numbers 8, 10, 11, 13, and 14) are arranged in circular formation like the blades of a propeller. A hinge region, built up from two helices and one strand (αE-β15-αF), connects the two domains by forming the bottom of a cleft. At the surface of the cleft, there is a cavity embedded in the large domain next to the carboxyl end of strand 1 of the β sheet. This cavity con-

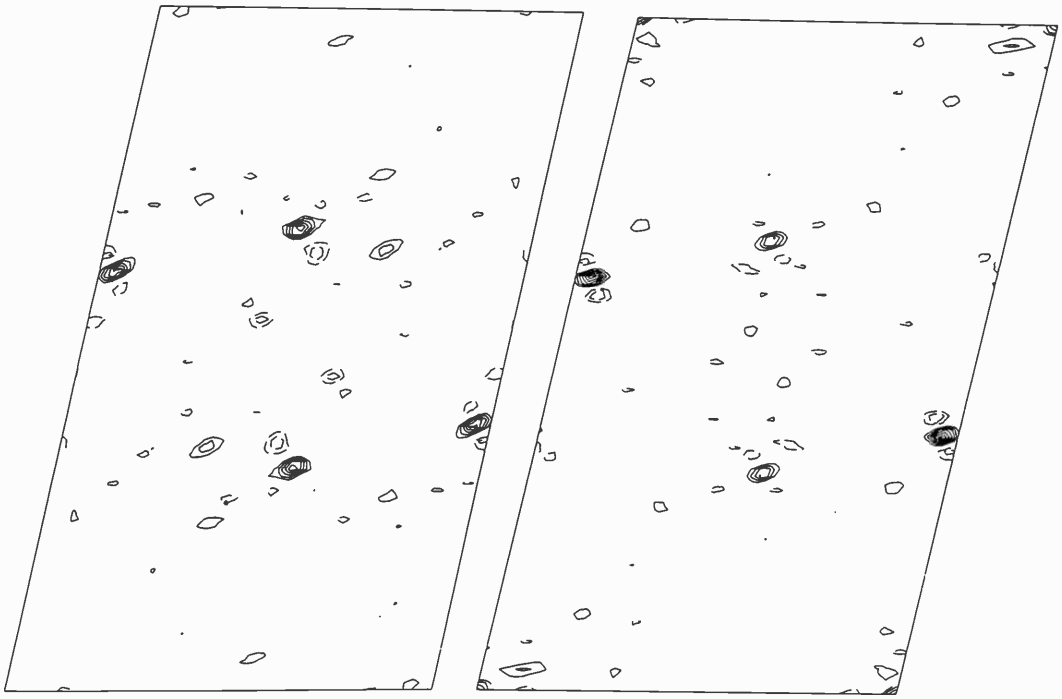

FIGURE 2 Harker section at v = 1/2. Peaks show pCMBS (*p*-chloromercuribenzene sulfate) sites in isomorphous difference Patterson map (*left*) and anomalous Patterson map (*right*).

tains the AdoMet binding site and is adjacent to the least variable PC dipeptide in which the cysteine is the active site nucleophile.

CONSERVED SEQUENCE MOTIFS AND SECONDARY STRUCTURE

Comparative analysis of available sequences of (cytosine-5)-methyltransferases shows the presence and order of the ten conserved motif sequence blocks (J. Posfai and R.J. Roberts): six well conserved motifs and four less conserved motifs. The variable region, located between conserved motifs VIII and IX, is responsible for the specificity of DNA recognition and the choice of the methylation target.

In the M·*Hha*I-AdoMet structure, the first eight conserved motifs are located in the large domain, and the variable region folds to form the majority of the small domain. The last two motifs run back to the large domains through the hinge region. The conserved motifs constitute most of the structures that surround the active site cleft. The six most conserved motifs form the core structure of the protein (Fig. 3). This includes the three α helices (αA, αE, and αG), the four central strands of the β sheet (6-4-3-1) and associated loop regions, named *l*-1A (between β1 and αA), *l*-3C (between β3 and αC), *l*-4D (between β4 and αD), and *l*-56 (between β5 and β6), and strand β14 in the small domain. The four central strands and the loop regions are part of the active site cleft.

Although the full motifs span β-strand, α-helices, and turns, the majority of the highly conserved or invariant residues occur in loops immediately adjacent to ordered secondary structures. Most of these invariant residues are clustered around the active site. In addition, two invariant residues, R272 from strand β14 in the small domain and E278 from helix αE in the hinge region, form an ion pair.

ADOMET BINDING POCKET

The presence of bound AdoMet in the crystals clearly defines the cofactor binding site. AdoMet binds to the large domain at the surface of the cleft next to the carboxyl end of the parallel strands of the β sheet. The binding of AdoMet involves the insertion of its adenosyl moiety into a pocket on the protein surface, whereas the methionine moiety points toward the solution. The β1-αA-β2 structural segment that recognizes AdoMet contains a highly conserved amino acid sequence F-x-G-x-G among (cytosine-5)-

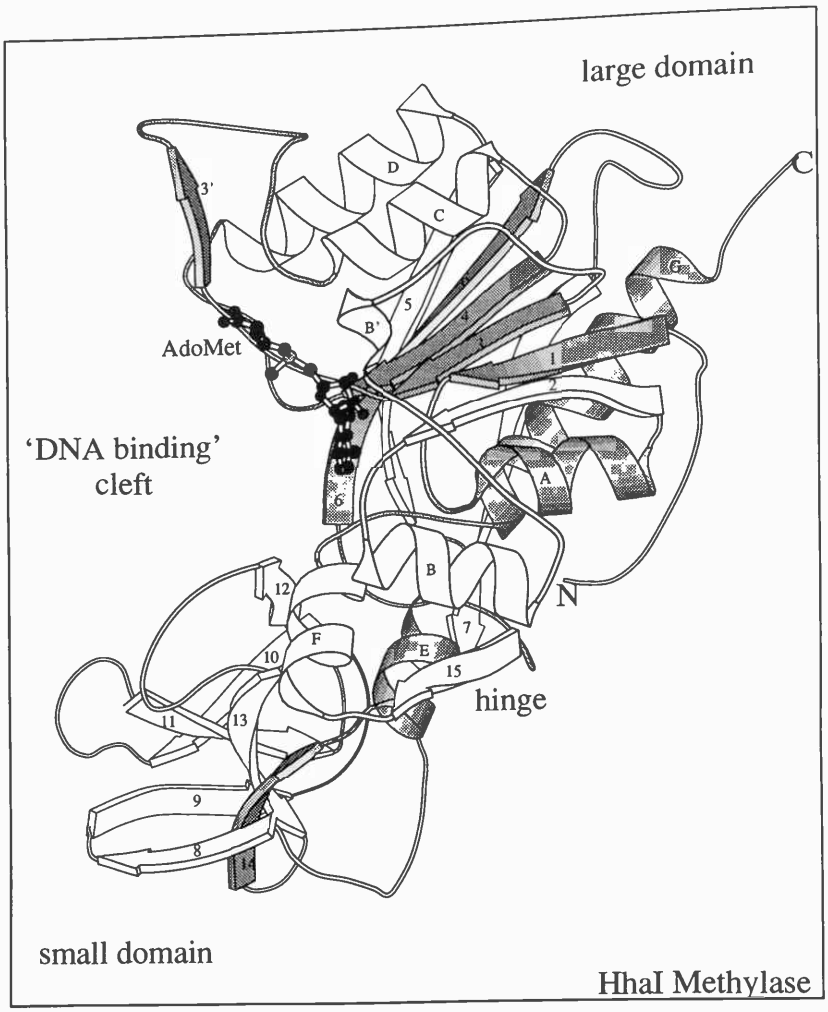

FIGURE 3 Ribbon representation showing the structure of M·*Hha*I methyltransferase complexed with AdoMet. The two structural domains of the molecule are seen clearly: large domain (aa 1–194, aa 304–327), small domain (aa 195–275), and hinge region (aa 276–303). The six highly conserved sequence motifs are shaded. The residues forming the secondary structural elements, N-(β1-αA-β2)-αB-(β3-αC-β4)-αD-(β5-β6)-β7-(β8-β9-β10-β11-β12-β13-β14)-(αE-β15-αF)-αG-C, are β1 (12:17), αA (23:31), β2 (33:40), αB (43:52), αB′ (43:52), β3 (74:78), β3′ (83:86), αC (101:110), β4 (115:120), αD (131:143), β5 (145:154), β6 (165:172), β7 (185:194), β8 (208:211), β9 (215:220), β10 (225:230), β11 (239:244), β12 (249:253), β13 (262:267), β14 (270:274), αE (276:283), β15 (289:292), αF (295:303), αG (308:323).

methyltransferases. The glycine-rich region forms a tight loop between strand β1 and helix αA, which appears to be crucial in positioning the adenine ring of AdoMet in its correct conformation in order to have close contact with the main chain of the protein framework. Large side chains at these glycine positions destroyed the general methylation capacity as shown by a mutational analysis of the SPR methyltransferase (Wilke et al., *EMBO J. 7:* 2601 [1988]). The conserved F18 along with surrounding hydrophobic amino acids P80 and L100 forms a hydrophobic platform on one side of the purine and ribose rings. W41 lies on the other side and its side chain nitrogen forms a hydrogen bond to N3 of the purine ring.

Although the sequence motif F-x-G-x-G had long been suspected of interacting with AdoMet, several other invariant residues were also involved. Two acidic residues, E40 and D60, interact directly with AdoMet. The side chain of E40, which is deeply buried in the hydrophobic pocket, is roughly perpendicular to the plane of the purine ring, with both

side chain carboxyl oxygens pointing to two purine nitrogens (N1 and N3). The side chain of D60 interacts with the positively charged trivalent sulfur atom, to which the transferable methyl is attached. N304, in the bottom of the pocket, forms a hydrogen bond to N6 of the purine ring. In addition, the backbone amino nitrogens of residues 41 and 61 form hydrogen bonds to O2 and O3 of the ribose ring. Most of the residues interacting with AdoMet are well conserved. F18, D60, P80, and N304 are absolutely conserved. E40 has only D variants, and L100 has M and V variants. W41 is not conserved.

CATALYTIC SITE

Cysteine residue 81, in the PC invariant dipeptide, is the key catalytic residue of M·*Hha*I. From the loop region outside of strand 3 (*l*-3C), C81 lies in the cleft next to the AdoMet binding pocket. Replacement of C81 with other amino acids abolishes catalytic activity (S. Mi and R.J. Roberts). The corresponding cysteine residue has recently been identified as the active nucleophile in M·*Hae*III (Chen et al., *Biochemistry 30:* 11018 [1991]).

In close proximity to the sulfur atom of C81, there is a histidine residue, H127, that is part of the loop region outside of β-strand 4 (*l*-4D). C81 forms a strong interaction with the side chain of H127, and this is probably the reason no mercury derivative was found to bind at this exposed cysteine in the substrate-free enzyme. We are not certain about the significance of this interaction since sequence alignment shows that H127 is not conserved. Interestingly, there are two invariant triplets around this active site: E119-N120-V121 from the last two residues of β-strand 4 and loop *l*-4D, and Q161-R163-R165 from loop *l*-56. There is a cascade of interactions among these two triplets, the P80-C81 region and the T250-L251 dipeptide in the small domain on the other side of the cleft.

DNA-BINDING CLEFT

Several observations suggest that the cleft between the two domains is the DNA-binding site. First, the presence of AdoMet and the nearby active nucleophile C81 within the cleft identifies this region as the active site. Second, the amino acids forming the surface of the cleft have a high degree of sequence homology between (cytosine-5)-methyltransferases, suggesting an important functional role for the cleft. Third, positive charges, capable of interacting with the DNA backbone, are contributed to the surface of the cleft by both domains. The triplet, Q-R-R, is well-conserved and lies on the surface of the large domain facing the cleft. This is a likely candidate for non-specific interactions with the sugar-phosphate backbone. Finally, the cleft itself is about 25–30 Å wide and 15–20 Å deep, large enough to accommodate double-stranded DNA without steric hindrance in a computer-generated model. The length along the bottom of the cleft, where the Q-R-R is located, is approximately 40 Å, potentially covering about 11 base pairs. This would correlate well with the 10 base pairs protected by the enzyme in the footprint experiment (S. Klimasauskas and R.J. Roberts). The length along the top of the cleft is only about 15 Å, which is the size of the recognition sequence. Thus, the residues at the bottom of the cleft could serve to orient the axis of the DNA helix, whereas those at the top of the cleft could directly interact with specific nucleotides.

We cannot identify any known DNA-binding motifs in the structure. In the large domain, a highly conserved 20-residue subdomain from loop *l*-3C, following the PC dipeptide and connecting to αC, is quite flexible in the model. The location of this region suggests that it could close off the cleft and wrap around the double-stranded DNA after substrate binding.

Both large and small domains apparently contribute to the DNA binding if it indeed occurs in the cleft, although the target specificity of DNA methylation is known to depend on the variable region, which constitutes the bulk of the small domain. Within the variable region, a small subregion, termed the target recognition domain (TRD), is believed to participate directly in sequence-specific recognition. Interestingly, the proposed position of the TRD for M·*Hha*I (centered around residues T250–L251) places it at the side of the cleft on the small domain. The residues of the proposed TRD are located in two regions in the structure: amino acids 231–240 (the loop connecting strands 10 and 11 then into strand 11) and amino acids 249–253 (strand 12). These two regions form the side of the cleft from the small domain, suggesting that these weakly conserved amino acids probably provide a "backbone" for DNA binding together with residues from the large domain. The specificity of individual target recognition would then be determined by the amino acids surrounding

and separating the conserved residues. Discussion of M·*Hha*I-DNA-AdoMet interaction must await high-resolution structures of the appropriate protein-DNA complexes.

Purification, Crystallization, and Preliminary X-ray Diffraction Studies of the M·*Hha*I-DNA Complexes

X. Cheng, K. McCloy [in collaboration with S. Klimasauskas, S. Mi, and R.J. Roberts, Cold Spring Harbor Laboratory]

Most DNA-binding proteins, such as several restriction enzymes and repressors whose structures have been solved, are dimers. Because DNA methyltransferases act as monomers, the structure of M·*Hha*I-DNA complexes will reveal novel properties unique to monomeric DNA-binding proteins. Crystals have been obtained for the ternary complex of M·*Hha*I, substrate analog *S*-adenosylhomocysteine, and a series of duplex oligonucleotides containing the recognition sequence GCGC: a blunt-end 12 mer and 11, 13, and 15 mers with one-base overhangs. The structure determination of the complex with the 13-mer DNA is currently under way. Crystals are monoclinic space group C2 and have unit-cell dimensions of a = 173.0 Å, b = 99.9 Å, c = 122.8 Å, and $\beta = 118^{o}$. A native data set has been collected and the structure should be readily determined using molecular replacement, with the refined structure of apomethylase as a search model. Concurrently, we are preparing conventional heavy atom derivatives by soaking and using iodinated oligonucleotides.

It has been shown that (cytosine-5)-methyltransferases form a stable, covalent complex with DNA containing 5-fluorocytosine. A proposed mechanism for M·*Hha*I methyltransferase predicts that this complex is a trapped reaction intermediate during catalytic methyl transfer, resulting from the formation of a Michael adduct between a cysteine residue (Cys-81) and the C-6 position of cytosine. The structure of this complex will be extremely informative in elucidating the details of the catalytic mechanism. Toward this goal, crystallization of a covalent adduct of 11-mer and 13-mer DNA containing 5-fluorocytidine with M·*Hha*I has been successful. We are crystallizing the covalent complexes with different lengths of oligonucleotides to obtain crystals with the highest possible resolution.

Replacement of the conserved cysteine (Cys-81) of M·*Hha*I methyltransferase with serine, arginine, histidine, and glycine abolished catalysis (S. Mi and R.J. Roberts). In addition, the glycine substitution was cytotoxic to *Escherichia coli* due to an extremely high affinity for DNA containing the recognition sequence. Purification and crystallization of the two mutant forms of M·*Hha*I (C81G and C81S) complexed with DNA have been initiated.

Structural Refinement of Bacteriophage T7 Lysozyme

X. Cheng [in collaboration with X. Zhang and F.W. Studier, Brookhaven National Laboratory, and J.W. Pflugrath, Cold Spring Harbor Laboratory]

Bacteriophage T7 lysozyme is a zinc amidase that cuts an amide bond on the bacterial cell wall, as well as being an inhibitor of T7 RNA polymerase. The crystal structure of a mutant T7 lysozyme (T7L), which deletes amino acids 2 to 5, has been further refined at 2.2 Å resolution. The initial coordinates for the T7L model were built from a 2.5 Å MIRAS (multiple isomorphous replacement with anomalous scattering) map (see last year's Annual Report). During this process, atomic models were used twice to improve the phases by phase combination with the MIRAS. Coordinates for the non-hydrogen atoms in T7L were initially refined against 2.5 Å native X-ray data by least-squares refinement, using the program X-PLOR. One hundred steps of Powell optimization smoothly reduced the R factor from 0.40 to 0.28 with no manual intervention, indicating the accuracy of the initial model. One cycle of simulated annealing refinement was then carried out, using initial and final temperatures of 3000K and 350K, respectively, and reduced the R factor to 0.24. A total of five rounds of model rebuilding using FRODO and least-squares refinement was carried out by adjusting the coordinates and temperature factors of individual atoms. Adjustments to the model were made from maps computed with Fourier coefficients (F_o-F_c) and ($2F_o$-F_c) and calculated phases. The resolution of the native data included was extended to 2.2 Å. Prominent nonprotein peaks in the (F_o-F_c) difference maps that are close to the protein surface and have rea-

sonable hydrogen bond lengths and angles were interpreted as water molecules and incorporated into the structural model. The occupancies of these waters were fixed at 1.0, and thus only the temperature factors and the coordinates were refined. Difference maps calculated after the fourth round of refinement revealed both incorrectly positioned solvent molecules and additional water molecules. A revised list of solvent molecules was then generated and subject to the final round of refinement. The current model has the entire sequence built in and 26 well-ordered bound water molecules and has unbroken backbone electron density from the amino to the carboxyl terminus in $(2F_o-F_c)$ difference Fourier maps. The R factor is 0.19 for 6991 reflections with $F_o > 2\sigma(F)$, and the root mean square deviation from ideal geometry for the 1514 atoms is 0.017 Å for bond lengths, 3.3° for bond angles, and 25.6° for the dihedral angle, all with good stereochemistry for the backbone torsion angles.

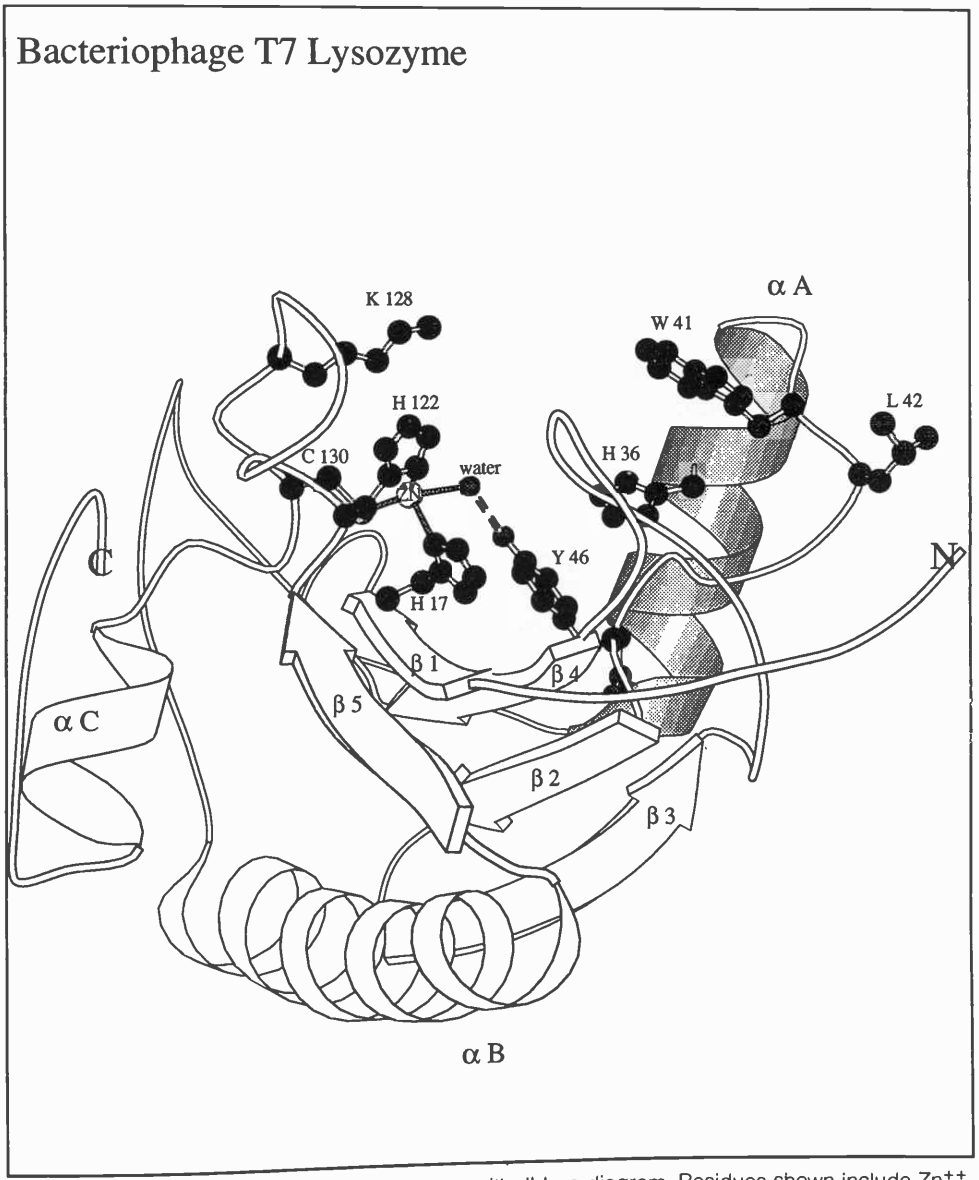

FIGURE 4 Side view of the cleft of T7 lysozyme with ribbon diagram. Residues shown include Zn^{++} ligands and several other amino acids that were the targets of site-directed mutagenesis. Helix αA, shown as shaded, appears to participate in both the amidase and the inhibition functions.

To identify residues critical for catalysis, the crystal structure was used to guide site-directed mutagenesis for several residues located within the cleft and/or on helix αA (Fig. 4), performed by Zhang and Studier at Brookhaven National Laboratory. The mutation at Tyr-46 (to Phe) and seven mutations at Lys-128 (to Asn, Gln, His, Ile, Met, Thr, or Trp) abolish amidase activity but have little impact on the inhibition activity, suggesting a crucial role for these two residues in catalysis. Helix αA appears to be involved in both functions. Mutants that abolish amidase activity but only affect inhibition slightly were obtained for His-36 and Trp-41, both located on αA and pointing toward the cleft. On the other hand, mutations that only affect inhibition activity greatly were found on two surface residues, Glu-31 and Leu-42. Since random mutations that reduce the inhibition activity were also found to be clustered on surface residues around helix αA and in the amino terminus (residues 1 to 8), these two nearby regions are likely to be involved in the interaction with T7 RNA polymerase.

PUBLICATIONS

Kumar, S., X. Cheng, J.W. Pflugrath, and R.J. Roberts. 1992. Purification, crystallization, and preliminary X-ray diffraction analysis of an M·*Hha*I-AdoMet complex. *Biochemistry* **31:** 8648–8653

NUCLEIC ACID CHEMISTRY

R.J. Roberts J. Earle-Hughes, S. Klimasauskas, S. Kulakauskas, S. Kumar, C.-L. Lin, D. Macelis, C. Marcincuk, S. Mi, G. Otto, K. Pepino, J. Posfai, D. Roberts, M. Wallace

A major research effort in this laboratory is directed at understanding the relationship between structure and function among the DNA (cytosine-5) methyltransferases (m5C-methylases). These enzymes, which accompany restriction enzymes in bacteria and are found free in both prokaryotes and eukaryotes, catalyze the transfer of a methyl group from *S*-adenosylmethionine to the 5-position of a cytosine residue in DNA. They differ as to the specific DNA sequence they recognize.

We have previously used biochemical, computational, and molecular biological approaches to study this class of enzymes. This year has been immensely productive as a result of a collaboration with Dr. X. Cheng (Macromolecular Crystallography Section), which has resulted in a structure at 2.6 Å for a complex between the *Hha*I methylase and its cofactor *S*-adenosylmethionine. This has been particularly gratifying as the *Hha*I methylase was first discovered in this laboratory in 1975.

The Structure of the *Hha*I Methylase

S. Kumar [in collaboration with X. Cheng, Cold Spring Harbor Laboratory]

We are delighted to report that we have solved the structure of M·*Hha*I bound to its cofactor *S*-adenosyl-L-methionine at 2.6 Å resolution. This structure, the first to be solved for any AdoMet-dependent methyltransferase, confirms the importance of the ten conserved motifs we had previously identified by computational methods that are present in nearly all m5C-methylases. The M·*Hha*I molecule is folded into two major domains, one small and the other large, with a deep cleft between them. The core of the M·*Hha*I structure is dominated by the most conserved motifs (1, 4, 6, 8, and 10), which cluster around the cleft and lie within the large domain. AdoMet and motif 4, the P-C motif that contains the

catalytic nucleophile Cys-81, are situated within the cleft, suggesting that this region is the active site. The size of the cleft is sufficient to accommodate DNA without steric hindrance, and basic residues capable of phosphate-backbone interactions are found on the small domain side of the cleft. The variable region between motifs 8 and 9, which determines the sequence specificity of the enzyme, constitutes the majority of the small domain. Motif 1, suspected to be part of the AdoMet-binding site on the basis of sequence similarity with other AdoMet-dependent methyltransferases, is shown to be directly involved in AdoMet interactions. In addition, we find that motifs 2, 3, and 10 are also involved in binding AdoMet. Using the knowledge gained from the M·*Hha*I structure, we are proceeding with experiments to delineate the functions of the conserved motifs, especially their roles in binding AdoMet and sequence recognition. Further details of the structure can be found in Dr. X. Cheng's report.

Biochemical and Crystallographic Analysis of the Ternary M·*Hha*I-DNA-AdoHcy Complexes

S. Klimasauskas

M·*Hha*I-COFACTOR INTERACTIONS

Recent studies (Kumar et al., *Biochemistry 31:* 8648 [1992]) indicate that at millimolar concentrations, M·*Hha*I tightly associates with its cofactor, AdoMet, and can readily be purified to homogeneity and crystallized in the AdoMet-bound form. We have further investigated several aspects of this binary complex. First, our data indicate that the complex is reversible. The addition of urea at concentrations over 2 M leads to the rapid release of bound AdoMet (several minutes at room temperature). Dissociation of the complex is also detectable under nondenaturating conditions: $t_{1/2}$ for the release of [^{3}H]AdoMet during dialysis of the labeled complex is approximately 2 hours at 25°C and 6.5 hours at 4°C. An estimate of the binding affinity using equilibrium microdialysis gives a K_d value of 13 μM (25°C). On the basis of the above results, we developed a large-scale procedure for the preparation of M·*Hha*I, free of cofactor.

M·*Hha*I-DNA INTERACTIONS

The potential of M·*Hha*I to bind DNA was investigated by gel-mobility-shift analysis. The binding to the 37-mer oligonucleotide duplex containing a unique nonmethylated or hemimethylated GCGC site is fast and quantitative. The interaction is enhanced in the presence of the cofactor analog, *S*-adenosyl-L-homocysteine (AdoHcy). The stoichiometry of the complex as determined by "titration" analysis is approximately 1:1. The complex is unstable under denaturating conditions such as boiling in the presence of 0.1% SDS or treatment with 4 M urea at 55°C. To determine the length of the DNA region contacted by the methylase, two synthetic 37-mer duplexes each containing the unique substrate sequence GCGC were footprinted using dimethylsulfate in the presence of the methylase and AdoHcy. The DNA substrates were identical in their sequences but differed in that their GCGC sites were hemimethylated on one strand or the other. The preformed complexes were briefly exposed to DMS, and, following strand cleavage, guanines protected by the bound methylase were identified by electrophoresis and autoradiography. The footprinting experiments show that interactions between M·*Hha*I and DNA in the major groove are localized within 10 base pairs, or approximately 34 Å, with the GCGC sequence residing in the center. This number is in good agreement with an estimated diameter for a monomeric protein of 327 residues.

CRYSTALLIZATION AND X-RAY DIFFRACTION OF TERNARY M·*Hha*I-DNA-ADOHCY COMPLEXES

This work was done in collaboration with X. Cheng. A series of synthetic self-complementary oligonucleotides were assayed for their ability to cocrystallize as ternary complexes with M·*Hha*I and AdoHcy. On the basis of our footprinting data, substrates of 11–15 nucleotides were chosen for these experiments. The oligonucleotides were annealed to form duplexes and mixed with the methylase and AdoHcy, and the ternary complexes were isolated by FPLC on an anion-exchange column. Large-scale preparations were performed that typically yielded 1–1.5 mg of the purified complexes at concentrations (10–15 mg/ml) suitable for crystallization. A sparse matrix screening method was used to search for initial crystallization conditions by the vapor diffusion technique in hanging drops. After initial crystals were observed, the conditions were refined to allow the optimal growth of crystals. To confirm that we were observing co-crystals, direct biochemical analysis was conducted on the crystals grown from the purified

complex with the 13-mer duplex DNA. Eleven crystals, previously used to collect diffraction data, were recovered from capillaries and were subjected to UV-spectral analysis, colorimetric protein determination, and urea treatment, followed by HPLC analysis of the released cofactor. The experimentally determined molar ratio of DNA:M·*Hha*I:AdoHcy is approximately 0.87:1:1.2, indicating an equimolar content of the components in the crystals.

Although all DNA fragments tested yielded crystals large enough for X-ray diffraction studies, there was significant variation in their diffraction power and stability. The methylase 15-mer crystals diffracted poorly beyond 10 Å resolution. The methylase 11-mer crystals exhibited much stronger diffraction limited at around 4 Å in the synchrotron beam, but in our hands, they did not grow larger than 0.35 mm. They also had a short life time in mother liquor as well as in the X-ray beam. Fortunately, the 12-mer and 13-mer methylase complexes under optimal conditions consistently produced large (0.4 x 0.4 x 1.2 mm) crystals of a diamond morphology that were stable in their original drops for several weeks. The diffraction limit varied from crystal to crystal, reaching in many cases 2.5 Å in the synchrotron beam. The 13-mer complex was subjected to further crystallographic analysis.

The crystals are of monoclinic space group C2 with unit cell dimensions a = 173.0 Å, b = 99.9 Å, c = 122.8 Å, and α = 118.0°. The space group identification is based on the systematic absence of reflections in precession photographs taken from four different layers of reciprocal space. The calculated volume of the unit cell is 1.87×10^6 Å^3.

To our knowledge, this study describes the first crystals of a specific ternary methylase-DNA-cofactor complex suitable for X-ray analysis. Isomorphous crystals of the complex with synthetic DNA containing a 5-iodouracil residue replacing one of the thymines were obtained. We are presently collecting diffraction data from both native crystals and heavy atom derivatives. Recently, isomorphous crystals of the ternary covalent complex with duplex DNA containing 5-fluorocytosine in the methylation target site have also been produced. This complex represents a mechanism-based covalently trapped reaction intermediate. Structure determination of these complexes should provide a detailed picture of substrate binding including the molecular mechanism of sequence discrimination. Because of the strong conservation of sequences among methylases, the results will be valuable in designing experiments to probe the functional details of all m5C-methylases.

M·*Hha*I Mutant Methylases

S. Mi

The family of m5C-methylases shares an overall common architecture: five highly conserved motifs about 10 to 20 amino acids long and five moderately conserved motifs. The P-C motif has universally conserved proline and cysteine residues and is the enzyme catalytic center. The cysteine residue in the P-C motif is involved in covalent bond formation with the target cytosine residue during catalysis. It was observed for the *Eco*RII methylase (M·*Eco*RII, recognition sequence CCWGG) that replacement of the conserved cysteine in the P-C motif with serine, valine, or tryptophan abolished catalysis. A glycine substitution both abolished catalysis and proved cytotoxic to *Escherichia coli* (Wyszynski et al., *Nucleic Acids Res. 20:* 319 [1992]). One hypothesis was that the mutant protein had a very high affinity for its target DNA, such that binding might interfere with transcription or replication. To investigate this possibility and to determine whether this cytotoxic phenomenon can be extended to other m5C-methylases, we have constructed several M·*Hha*I mutants in the P-C motif by substitution of the conserved cysteine (Cys-81) with four different amino acids. Replacement by arginine, glycine, histidine, and serine all result in loss of methylase activity. Interestingly, the glycine mutant is cytotoxic in *E. coli*.

A series of studies was carried out to establish the cause of the cytotoxicity of the Gly-81 mutant methylase. It was found that the glycine mutant methylase as well as the other three mutants showed no sensitivity to 5-azacytidine, indicating that the protein has no enzyme activity. This was confirmed by the complete sensitivity of plasmid DNAs encoding the mutant methylases to digestion in vitro by the *Hha*I restriction endonuclease. Purified Gly-81 mutant protein could only be isolated as a protein-DNA complex from normal *E. coli* cells. However, when the Gly-81 mutant protein was isolated from cells co-expressing the *Sss*I methylase (recognition sequence CG), it was now well-behaved and could be isolated free of DNA. When subjected to gel-shift competition assays to assess its DNA-binding properties, the

TABLE 1 Kinetic Constants for the Methyltransferases

Methyltransferase	$t_{1/2}$ (min)	$k_{off} \times 10^{-3}$ (min^{-1})	$K_d \times 10^{-9}$ M
Cys-81 (wt)	11	65	1.25
Arg-81	1.5	481	11
Gly-81	568	1.2	0.45
His-81	0.63	1000	20
Ser-81	69	10	1.69

Gly-81 mutant exhibited a much higher affinity for its target DNA sequence than the wild-type enzyme. The k_{off} rate is 50-fold slower and the K_d is 3-fold smaller than the values for the wild-type protein. The dissociation rates and the K_d values of the wild-type and mutants are presented in Table 1.

In summary, replacement of the catalytic cysteine residue by glycine results in a mutant methylase that binds DNA extraordinarily tightly. If unchecked, this tight binding interferes with normal cellular metabolism and causes cytotoxicity in *E. coli*.

Genetics of Restriction-Modification

S. Kulakauskas

FORMATION OF DIMETHYLCYTOSINE IN VIVO

The *Eco*RII (or the *E. coli dcm* methylase) and *Mva*I methylases both modify the second cytosine residue in the CCWGG sequence but differ in the precise modification introduced. *Eco*RII produces C5-methylcytosine, and *Mva*I produces N4-methylcytosine. In vitro, the *Mva*I methylase can modify a substrate that already carries a methyl group at the C5 position of the target cytosine. The result of this double enzymatic methylation is the formation of a novel modified base, N4,C5-dimethylcytosine (4,5mC) (Butkus et al., *Nucleic Acids Res. 13:* 5727 [1985]). We are investigating the formation of 4,5mC in vivo and its consequences for the growth of *E. coli*. We assume that 4,5mC should occur in a *dcm*$^+$ strain of *E. coli* carrying the *Mva*II methylase gene on a plasmid. We analyzed plasmid DNA in strains expressing different levels of the *Mva*I methylase and were unable to detect 4,5mC, although 5mC and 4mC were readily detectable (S. Klimasauskas, unpubl.). This result suggests that 4,5mC residues, if they form, are effectively removed from DNA.

We tested for the induction of the *E. coli* SOS system after transformation with plasmids carrying the cloned *Mva*I methylase. Strains containing a fusion of the β-galactosidase gene to one of the SOS genes, *din1*, were used. Induction of β-galactosidase expression was detectable only after transformation of this strain with a plasmid carrying an intact M·*Mva*I gene. Either a *dcm6* mutation in the chromosome or a point mutation in the M·*Mva*I gene prevented β-galactosidase induction. The SOS response in *E. coli* is known to be induced by double-strand breaks in DNA that may occur because of excision of 4,5mC in vivo.

We also tested the effect of mutations in the SOS genes on the viability of *dcm*$^+$ cells expressing the *Mva*I methylase. A set of strains with various mutations in repair genes were transformed with plasmids carrying M·*Mva*I. Strains carrying *recBC*, *recBC sbcB recF*, *mutS*, or *dam* mutations show normal levels of transformation. In contrast, strains carrying *ung1*, *recA*, *recD*, *recJ*, *mutH*, or *uvrB* mutations either fail to produce viable transformants or small colonies appear after 48 hours incubation. A plasmid containing an inactivated M·*Mva*I gene transforms these strains normally. These results suggest that synthesis of 4,5mC takes place in vivo, but this unusual base is efficiently removed from DNA. This process of repair is accompanied by induction of the SOS genes.

SEQUENCE OF THE *Hpa*II RESTRICTION ENDONUCLEASE GENE

The *Hpa*II restriction-modification system from *Haemophilus parainfluenzae* recognizes the DNA sequence CCGG. A 10-kb DNA fragment carrying the active methylase gene had previously been cloned and the sequence of the methylase gene determined. However, clones carrying the methylase gene did not express the cognate restriction endonuclease (Card et al., *Nucleic Acids Res. 18:* 1377 [1990]). Separately, a 5-kb fragment of *H. parainfluenzae* DNA was cloned that expresses both the *Hpa*II methylase and restriction endonuclease activities (S. Kulakauskas and A.A. Lubys, unpubl.). The region was sequenced and a 1074-bp open reading frame was found downstream from the methylase gene. It overlapped the methylase gene by 8 bp. Deletion of the DNA segment containing this reading frame abolishes restriction endonu-clease activity, suggesting that it encodes the *Hpa*II restriction endonuclease.

Integration Host Factor: A DNA-binding Protein in *Escherichia coli*

D. Roberts

Integration host factor (IHF) is a site-specific DNA-binding protein in *E. coli*. It was discovered originally because of its essential role in integration of bacteriophage λ and has since been shown to influence gene expression, site-specific recombination, and replication in a variety of bacterial systems. IHF is a member of a highly conserved family of small basic proteins in prokaryotes. It has been suggested that these proteins play a role in DNA structure and organization analogous to histones in eukaryotes.

We undertook an analysis of IHF with the intention of mapping the regions of the protein that are critical for DNA binding, site specificity, and DNA bending. To do this, mutants of IHF were isolated either by random mutagenesis (EMS) or by site-directed mutagenesis using specific oligonucleotides. IHF is a heterodimer, and both subunits were mutagenized. In addition, the two subunits, IHFα and IHFβ, are highly homologous, and hybrid proteins were generated by substituting segments of IHFα and IHFβ. The mutants were analyzed for function in a variety of assays. They were tested for their ability to support repression of a Tn*10* transposase:*lacZ* fusion, replication of the plasmid pSC101, and growth of several phages (λ, λ derivatives, and Mu).

IHFα and IHFβ are different. Amino acid changes in the putative DNA-binding arm of IHFα can completely eliminate IHF activity, whereas identical changes in IHFβ have no effect. This result suggests that IHFα and IHFβ have different roles in DNA recognition or binding, with IHFα being much more important. This is further suggested by the study of hybrid proteins. IHFβ is much more tolerant of substitutions; one third of IHFβ could be replaced by IHFα without eliminating IHF activity. In contrast, IHFα is quite sensitive to substitutions, and most substitutions, even of 10–20% of the protein, destroyed IHF activity.

Basic amino acids present in the carboxy-terminal 15% of IHF are important. Loss of only one or two of these basic residues has little effect on activity. Loss of three or four greatly reduces IHF function. IHFα and IHFβ have little homology in this region except for the basic amino acids. The two regions are functionally equivalent and either can provide full IHF activity in hybrid protein.

Protein Sequence Motifs

J. Posfai, C.-L. Lin

Short stretches of amino acid sequence (motifs) that correlate with protein function are useful tools to help in the interpretation of newly determined DNA and protein sequences. Their presence can often lead to accurate prediction of the function for hypothetical products. Our goal is to provide semi-automated methods for the discovery of such motifs with predictive value and to automate the maintenance of a database of motifs.

Sequences that may share some common function (e.g., catalyzing the same reaction or binding the same substrate) are retrieved from DNA and amino acid sequence databases by routines that are based on interpretation of database annotations. This initial step is followed by format conversions and translations. A combination of automatic and manual pruning eliminates uninformative fragments as well as duplicate copies of sequences. A clustering algorithm is used to establish sets of functionally related sequences. Conserved regions of these sequence groups are then determined by alignment, based on algorithms similar to those described by Smith et al. (*Proc. Natl. Acad. Sci. 87:* 118 [1990]). Different representations (consensus patterns, regular expressions, sequence blocks, weight matrices, neural nets) of conserved sequences are created and considered initial motifs. These are then used to search the Swiss-Prot database. If they are found to occur in sequences other than those initially used to define them, the hits are examined for true positives, false positives, or unknowns. If necessary, the motifs can then be refined to provide greater predictive value.

A fundamental feature of all our algorithms is that they look upon sequences as chains of overlapping amino acid triplets that may span distances of average secondary structural units. This allows the recognition not just of conserved residues of independent positions, but also of higher-order conserved patterns (e.g., amphiphilic helices), as well as interactions between nonneighboring residues that have remained stable over evolution. When designing and coding our programs, high priority is given to innovative visual presentations of results and to user-friendly interfaces. We are presently integrating the main parts of our database management utilities and developing compatible links to other widely used sequence analysis programs like BLAST, FASTA, and BLOCKS.

REBASE: The Restriction Enzyme Database

D. Macelis, J. Earle-Hughes, R.J. Roberts

The restriction enzyme database, REBASE, continues to grow and provide primary information about restriction enzymes to researchers worldwide. We have now completed the transition from the ORACLE data management system to SYBASE. This has greatly streamlined our operation. The database is distributed in more than 20 different formats each month, and there has been extensive use of our anonymous FTP service. During the last year, the number of known restriction enzymes has grown to more than 2300, with 186 different specificities represented.

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PROTEIN KINASE STRUCTURE AND FUNCTION

J. Kuret P. Kearney G. Carmel
A. Sessler B. Leichus
P.C. Wang A. Vancura

Our work focuses on two protein kinases: casein kinase-1 and the cAMP-dependent protein kinase. Our progress in applying the techniques of X-ray crystallography and molecular genetics to the study of protein kinases is described below.

Casein Kinase-1

P.C. Wang, A. Vancura, B. Leichus

Casein kinase-1 (CK1) is a ubiquitous eukaryotic protein kinase that phosphorylates acidic substrate recognition sequences efficiently. Once considered a single entity, it is now known to consist of subspecies that together comprise a distinct branch of the eukaryotic protein kinase family. Family members identified to date consist of a highly conserved, approximately 290-residue amino-terminal catalytic domain, joined to a carboxy-terminal region that is not conserved between family members and that varies in size from 40 to 180 amino acids. Although the full role of the carboxy-terminal domain is not understood fully, part of its function is to target the enzyme to specific regions of the cell.

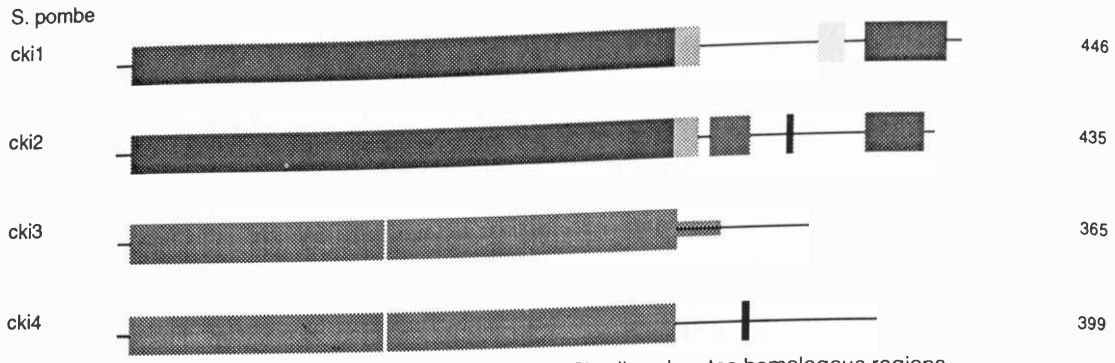

FIGURE 1 The CK1 family from *S. pombe*. Shading denotes homologous regions.

This year we isolated the complete catalog of CK1 genes from the yeast *Schizosaccharomyces pombe* (Fig. 1). The predicted gene products range in size from 43 to 52 kD and follow the structural organization described above. As in the *Saccharomyces cerevisiae* system, two of the isoforms contain a consensus prenylation site at their carboxyl termini (cki1/2), whereas the other two (cki3/4) contain glutamine-rich regions.

To examine the biochemical properties of these enzymes, each was expressed in bacteria, purified to homogeneity, and characterized enzymologically. A surprising result was that *S. pombe* CK1s are dual specificity kinases (i.e., they phosphorylate tyrosine as well as serine and threonine residues). Although each CK1 form can autophosphorylate on tyrosine, only cki3/4 are capable of *trans* tyrosine phosphorylation to exogenous peptide substrates in vitro. In the coming year, we plan to investigate the role of tyrosine phosphorylation in CK1 function.

Subcellular Distribution of Casein Kinase-1

A. Vancura, A. Sessler

CK1 is implicated in a wide variety of cellular functions. In nuclei, it phosphorylates and inactivates T antigen (an initiator of DNA replication), as well as p53, a tumor-suppressor protein. In addition, it binds and possibly regulates kinesin, a mitotic motor. Cytoplasmic CK1 is activated in response to growth factor treatment and is capable of phosphorylating the cortical cytoskeleton. In addition to its role in normal cells, CK1 has been implicated in the pathogenesis of Alzheimer's disease through the phosphorylation of τ protein, the major component of neurofibrillary tangles.

To identify the individual CK1 isoforms involved in nuclear or cytoplasmic regulation, we examined the subcellular localization of each of the CK1 isozymes of *S. cerevisiae* and *S. pombe*. We find that in both of these organisms, the prenylated isoforms cofractionate with RAS and localize exclusively with the plasma membrane. Membrane association is mediated by a pair of carboxy-terminal cysteine residues that are probable sites of prenylation. These forms appear to transduce extracellular signals: In the yeast system, the signal is the osmolarity of the growth medium. A second pair of CK1 isozymes is more broadly distributed, being found primarily in the nucleus and the plasma membrane.

We hypothesize that CK1 functions at two levels. The plasma membrane forms may act in a classical kinase cascade mechanism. Here, CK1 probably receives input from an osmolarity "receptor" or sensor and dispatches output to intracellular kinases to coordinate a biological response. Nuclear CK1 may also be a component in a signal transduction pathway. Yet, the unusual phosphate-directed substrate selectivity of CK1 suggests a second mechanism of regulation we call "transistor amplification" (in electronics, a transistor amplifies a weak current by superimposing it on a larger current). A protein phosphorylated at a single site will become a substrate for CK1. Additional phosphates will improve CK1-mediated phosphorylation still further, resulting in the phosphorylation of the substrate to high stoichiometry. In this analogy, the first phosphorylation event, which may be catalyzed by a distinct protein kinase, is the weak current, whereas the multiple phosphorylation catalyzed by CK1 is the strong

current. The information carried by the first phosphorylation event is amplified by the large flux of phosphate produced by CK1. Transistor amplification is an efficient mechanism: A typical electronic transistor produces a 100-fold gain (amplification) in current. Nuclear CK1 may achieve similar amplifications without the need of a cascade mechanism.

Casein Kinase-1 Tertiary Structure

G. Carmel, P. Kearney [in collaboration with X. Cheng, Cold Spring Harbor Laboratory, and R. Sweet, Brookhaven National Laboratory]

Catalytically, CK1 differs from most other protein kinases in that its substrate selectivity appears to be directed toward phosphate groups rather than unmodified amino acids. Although CK1 can phosphorylate synthetic peptide substrates containing glutamic or aspartic acids at position –3 or at position –4 (relative to the position of a phosphorylatable serine or threonine residue), substitution of those carboxylic acid residues with phosphoserine yields a dramatically superior substrate. How CK1 recognizes phosphopeptides is unclear and may differ substantially from the well-characterized interaction between the cAMP-dependent protein kinase and its inhibitor protein.

To place CK1 enzymology within a structural framework, we initiated an effort to determine the crystal structure of a representative member of the CK1 family. We began with one of the *S. pombe* clones described above and engineered it so that it now encoded only the catalytic domain of the enzyme. Next, we produced large amounts of enzyme through heterologous expression in bacteria and purified the product to near homogeneity. Finally, the preparation was concentrated and subjected to crystallization trials, the results of which are shown in Figure 2. In the absence of substrate, CK1 crystallizes as rhombohedral prisms (Fig. 2A) up to 0.3 x 0.3 x 0.3 mm in size that diffract to medium resolution (3.2 Å). By examining the systematic absences of reflections in our diffraction data, we determined that the packing of protein molecules in the crystal (i.e., the space group) had $P2_12_12_1$ symmetry. In the presence of MgATP, CK1 crystallizes in two different crystal forms. The first form (Fig. 2B) emerged as natty needles with dimensions 1.0 x 0.1 x 0.1 mm and that diffract to 2.7 Å resolution. Again, assignment to space group $P2_12_12_1$ was made on the basis of systematic absences in our diffraction data. The second binary form emerged as trigonal prisms with dimensions 0.8 x 0.5 x 0.5 mm (Fig. 2C) and that diffract to high resolution (~2.0 Å). Assignment to space group $P3_121$ (or its enantiomorph) was made on the basis of precession photography. Two isomorphous heavy atom derivatives of this trigonal crystal form have been prepared.

Our strategy for the coming year is to use these derivatives to solve the phase problem (by the classical method of multiple isomorphous replacement)

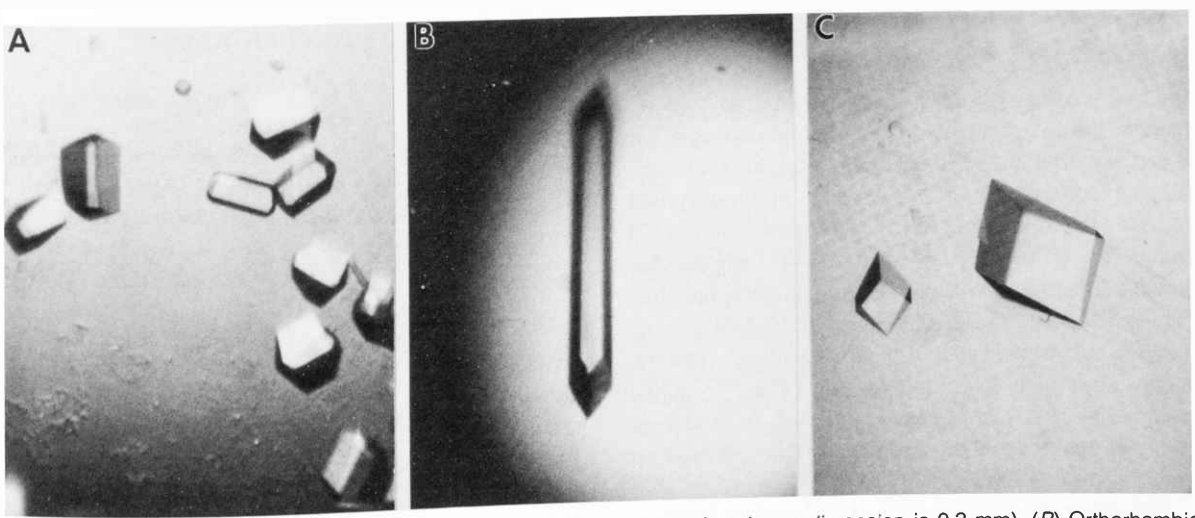

FIGURE 2 CK1 crystals. (*A*) Orthorhombic crystals of unliganded CK1 (maximum dimension is 0.3 mm). (*B*) Orthorhombic binary form (maximum dimension is 1 mm). (*C*) Trigonal binary form (maximum dimension is 0.6 mm).

and to deduce the tertiary structures of both enzyme and ligand. Once a model structure is obtained, the crystal structures of the needle binary complex and of the unliganded enzyme will be solved by the molecular replacement method. Together, the structures will reveal the location of key residues involved in nucleotide and protein substrate recognition, as well as the nature of the conformational changes that occur upon binding of nucleotide substrate.

The "Open" Conformation of the cAMP-dependent Protein Kinase

J. Kuret, G. Carmel [in collaboration with J. Pflugrath, Cold Spring Harbor Laboratory, and R. Sweet, Brookhaven National Laboratory]

Years ago, using methods similar to those described above for CK1, we initiated a structural study of TPK1, the catalytic subunit of the cAMP-dependent protein kinase (PKA) from *S. cerevisiae*. Over the years, we have prepared nearly 0.5 g of purified protein and have grown more than 1000 single crystals of the unliganded form of TPK1. Eager to complete this project and get out of the cold room, we obtained the coordinates of mammalian PKA and gave them to our crystallographer. It worked like a charm: The two structures were so similar that "solving" the structure of TPK1 appeared trivial.

When the structure of TPK1 is complete, what will it look like? Well, it will look a lot like the preliminary model illustrated in Figure 3. There, the partially refined catalytic core of TPK1 (2.9 Å resolution) is compared to the highly refined 2.2-Å structure of fully liganded, mammalian PKA. Despite being separated by over 500 million years of evolution and having only 50% identity in amino acid sequence, the two enzymes appear remarkably similar in tertiary structure. First, the catalytic core of each enzyme consists of an 85-residue, amino-terminal domain (the shaded region in each panel of Fig. 3) linked to a larger carboxy-terminal 183-residue domain (illustrated without shading in each panel of Fig. 3) that contains the peptide-substrate-binding site (peptide substrate is shown in dark shading). Second, a phosphorylated threonine residue (Thr-197 in the mammalian PKA numbering system) is found in identical positions of the carboxy-terminal domain of each enzyme.

Assuming the conformational differences between TPK1 and mammalian PKA result from the absence or presence of substrates, what can we learn about the structural consequences of ligand binding? In its open conformation, the ligand-binding surfaces of PKA are exposed to solvent and are accessible to substrates (Fig. 3A,C). When substrates bind, the amino-terminal domain rotates relative to the large domain along a hinge connecting the two domains, resulting in closure of the nucleotide-binding loop over the nucleotide substrate. The movement is a simple rigid body movement that involves a pivot point located in the small domain (Fig. 3B,D). The conformation of the carboxy-terminal domain appears to be unaffected by ligand binding.

Does the autophosphorylated threonine residue play a role in substrate binding? In the open conformation, phosphothreonine makes hydrogen bonds to three nearby residues, all of which are located in the carboxy-terminal domain (the side chains for two of these, Arg-165 and Lys-189, are shown in Fig. 3A but omitted in the other panels for clarity). Upon substrate binding, a histidine residue in the small domain (His-87) moves approximately 7 Å to make a new hydrogen bond with the phosphothreonine, thereby aligning and stabilizing the ternary complex. The contribution to stability is modest: Mutation of the phosphothreonine to an alanine (the side chain of which is incapable of forming hydrogen bonds) reduces the apparent second order rate constant for substrate/enzyme association only threefold.

In the coming year, our preliminary model of TPK1 will be refined so that it and mammalian PKA can be compared in detail.

S100β Is Not a Protein Kinase

J. Kuret, R. Hsu

Robbie Hsu was angry. He had just wasted another week preparing S100β from a pile of rat brains that looked like an Alcino Silva experiment gone horribly wrong. Worse still, he was unable to reproduce the notoriously fickle crystallization conditions for the purified protein. There had to be some way of improving the yield and quality of the protein or the project would be doomed. Then it hit him. An expression strain for S100β would solve the problem. The strain was constructed and the results were dramatic. Pure, active protein could be isolated in large amounts in only 3 days. Better still, the protein crystallized in a form that diffracted to higher resolution than the original material isolated from animals.

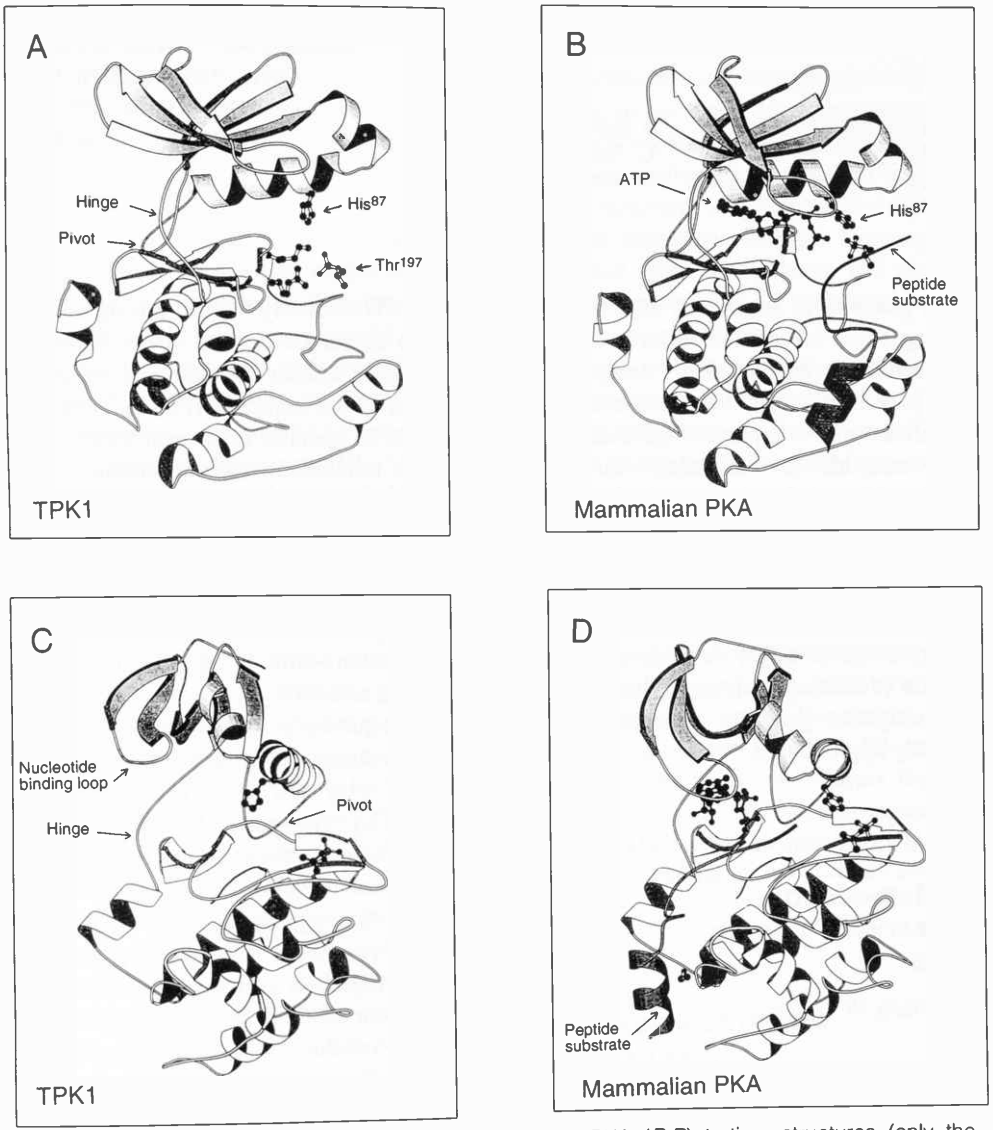

FIGURE 3 Comparison of TPK1 (*A,C*) and mammalian PKA (*B,D*) tertiary structures (only the catalytic cores are illustrated). Panels *B/D* are related to panels *A/C* by a 90° rotation around the ordinate axis. These images were generated by the program MOLSCRIPT, written by Per Kraulis.

Progress with S100β crystallography, which takes full advantage of our expression strain, is summarized in the Macromolecular Crystallography Section.

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COMPUTATIONAL MOLECULAR BIOLOGY

T. Marr L. Catapano, W. Chang, S. Cozza, E. Cuddihy, W. Li, D. Lombardi, J. Salit, C. Reed, M. Zhang

Research in our group is in an area of what has become known as genome informatics. This is a broad area including databases and sequence analysis. In our group, we primarily study new database technologies for application to comparative genome analysis and new methods for detecting functional regions in DNA and protein sequences. This is in anticipation of the time when DNA sequencing technology will greatly surpass the ability to perform meaningful experiments on even a relatively small subset of potentially important sequences. Thus, it will be necessary to have highly accurate computational methods for predicting biologic content buried within long, contiguous genomic sequences. This time is very rapidly approaching.

Automated Methods for Finding Conserved Patterns in Sequences

L. Catapano, W. Chang, W. Li, T. Marr

We are studying new computational methods for discovering conserved sequence patterns in collections of functionally related sequences. The long-term goal of this work is to see if we can develop the means of predicting the function of genes and gene products from the linear sequence alone. We have been studying a variety of collections of protein sequences, cyclins, kinases, proto-oncogenes, and so on. In the proto-oncogene collection, we discovered an intriguing sequence. It is intriguing because it was detected in a group of proteins involved in a signal transduction pathway, involving a receptor (platelet-derived growth factor receptor, PDGFR), several cytoplasmic tyrosine kinases (of the src family), and a nuclear transcription factor (p53). We have been investigating this amino acid sequence, IITxxxxxGNLL, the distribution of proteins in which this sequence is present, and the location of the sequence within a protein. We initially located the signal in the catalytic domain of abl kinase and at the boundary of one of five highly conserved domains in p53. Conserved versions of the sequence (i.e., IVTxxxxxGSLL) were found to be specific to several subfamilies of protein tyrosine kinases. In the abl subfamily, the sequence exists in the catalytic domain of dash and arg in addition to abl. In the PDGFR class of receptor tyrosine kinases, the sequence appears at the carboxy-terminal end of the kinase I domain (bordering on the kinase insert region) in PDGFR-α and PDGFR-β, macrophage colony-stimulating factor receptor (CSF-1 receptor), and c-kit.

The sequence is also found in the kinase domains of all members of the src family (src, fgr, fyn, hck, lck, lyn, yes) and several src-related kinases (srk, CSK, STK). This particular pattern has not yet been detected in any other sequence outside of the above familiar cast of characters, all of whom are implicated in tumorigenic activity. The identities of the central five amino acids are not conserved, but this variable region is consistently exactly five residues. At least one of these five amino acids is a phosphorylatable residue, with few exceptions. Computer analysis of secondary structure indicates that the signal invariably appears on the surface of the protein, consistent with the possibility that this signal is a phosphorylation site. A single mutation in a conserved version of this sequence in src has been shown to activate src's transforming capacity (Kato et al., *Mol. Cell. Biol. 6:* 4155 [1986]); in human p53, mutation in this sequence is polygenetically associated with breast cancer tumors. Thus, there is reason to believe that this sequence is involved in an important genetic function, possibly (most optimistically) indicating that the set of proteins within this sequence interact with a common molecule. Very little is known about the sequence dependency of phosphorylation activity. We are presently developing collaborations with experimental scientists to examine further the consequences of mutating this signal in an effort to elucidate its function.

Genome Topographer: Computer Software for Integration and Elaboration of Comparative Genomic Data

S. Cozza, E. Cuddihy, J. Salit, E.C. Reed,
W. Chang, T. Marr

Significant progress has been made in the ongoing process of genome-level molecular characterization of the genomes of human and many model organisms. There are nearly completely ordered YAC and COSMID contigs of several human chromosomes as well as the first complete sequence of an entire budding yeast chromosome (chromosome III, 315 kb). These are examples of some early remarkable successes of the international genome program. A growing tendency within the genome community is to develop specialized databases centered on specific organisms (e.g., ACeDB, GDB, FlyBase, *Esherichia coli*). These are very important sources of richly annotated (e.g., developmental information, gene disruption information) collections of genomic information, yet each of these databases requires its own idiosyncratic collection of software and hardware to be able to view data within the database. This often presents a significant barrier to using these databases because it is difficult to learn how to use many different software packages effectively, running on many different types of computers. Those who study gene function exploit evolutionary conservation extensively in their investigations; they are not limited to studying genes in any one particular organism. With these facts in mind, our research has explored the following questions: Can collections of apparently disparate genomic data be organized into what appears to the user as one computer program/database? Can such a system be developed so that it is capable of being continuously updated, with regard to both new understanding concerning existing data and new data types arising from new technology developments?

We have developed an advanced prototype of an object-oriented computer system that uses a largely organism-independent underlying data model to accomplish unification of genomic data, from low-resolution maps to sequence, across the major eukaryotic model organisms. The system is highly interactive, using intuitive graphical displays, icons, pull-down menus, and drag-and-drop paradigms for user actions, allowing the user to retrieve reference data, create new data using object-based editors, or enhance the annotations of existing data, by making links between independently derived data, for example. We are currently extending the system to support sequence similarity searching (on a network-based, socket-accessible, nonredundant sequence database whose structure is optimized for searching), tools to discover conserved elements in groups of functionally related protein sequences, and tools to find combinatorial collections of short conserved elements in the sequence databanks.

We are working with the Stanford budding yeast group to construct the first integrated database of the budding yeast and the fission yeast. These two well-studied yeasts are as far apart from each other as each is from the human; detailed comparison of sequences and genetics of these two organisms will yield much information on molecular evolution, since it is commonly observed that although there are many functional homologs existing between the two yeasts, many, if not most, of the homologies are not detectable at the DNA sequence level by standard sequence analysis methodologies. The system software is programmed using the Smalltalk-80 programming language and the GemStone Object Database Manager and runs uniformly on the host operating systems of most popularly used computers, including Macs, PCs, and Unix machines.

Large-scale Structure of Genomes

W. Li, M. Zhang, T. Marr

We are interested in how information is organized in DNA sequences using various mathematical and statistical analysis. In particular, following up our recent discovery that correlations far beyond their neighborhoods exist in some DNA sequences (W. Li, *Int. J. Bifurcation and Chaos 2(1):* 137 [1992]; W. Li and K. Kaneko, *Europhysics Letters 17:* 655 [1992]). We are gearing up to study large-scale correlation structures in completely sequenced chromosomes. This large-scale organization of the information in a primary sequence should reflect the overall three-dimensional structure of the chromosome. On that aspect, our studies will provide new insight into the structural organization of chromosomes. We plan to study the distribution and statistical features of the in-

itiation sites for replication and transcription in the sequence of *Saccharomyces cerevisiae* chromosome III. It is hoped that this distribution will aid our understanding of the parallel processes of replication and transcription.

To study long-range correlations in DNA sequences, we performed a statistical analysis of the entire genomic sequence of yeast chromosome III (M. Zhang and T. Marr, in press). Many biologists believe that knowing an adenine at one position should not change the likelihood of finding another adenine far apart. It turned out that knowing an adenine at one position actually increases the likelihood of finding another adenine even 1000 base pairs apart.

More specifically, our results showed the following. (1) Long-range correlations indeed exist: A like pair of nucleotides (A-A, C-C, etc.) tends to be positively correlated and an unlike pair (A-C, G-T, etc.) tends to be negatively correlated. (2) Global strand symmetries also exist: The probability of finding nucleotide x some distance downstream from nucleotide y is approximately equal to that of finding Y some distance downstream from X (where X and Y are complementary to x and y, respectively). These findings imply that duplication and inversion events (in roughly equal occurrence) have played a major role in genome evolution.

Computer Detection of Protein-coding Regions

W. Li, M. Zhang, T. Marr

One of the more challenging problems in computational molecular biology is to distinguish noncoding segments of a DNA sequence from the coding segments. These "coding measures" usually take into account certain regularities in the coding region that are absent in randomly generated sequences (e.g., the existence of the periodicity of three; the difference of nucleotide usage in three codon positions). Computer algorithms for coding-region recognition based on these coding measures actually distinguish coding regions from a randomly generated sequence, instead of distinguishing them from introns.

During our studies of long-range correlations in DNA sequences, we observed that some introns have their own regularity when compared with a random sequence: They tend to have longer correlation lengths. The preliminary result was derived from a small sample set of human DNA sequences. We plan to carry out a comprehensive study of the correlation structure in both coding and noncoding regions on a much larger data set. Specifically, we will calculate the two distributions of correlation lengths for both exons and introns (by calculating a correlation measure called "mutual information" for each distance; the correlation length is the distance at which the correlation is more or less zero). The degree of overlap of these two distributions will provide a conclusive answer as to whether the correlation length can be used as a useful "noncoding measure."

We have been exploring another approach to the problem of finding coding regions. We began with the study of exon recognition. We divided the problem into two subproblems: (1) Given both ends of a gene (maybe only approximately), how can we locate all of the introns? (2) Given genomic sequence alone, how can we find all of the initiation/termination sites? We took the fission yeast as an initial model; fission yeast has more introns and less conserved splicing signals than the budding yeast. The lengths of introns/exons are usually much shorter than what is required by many existing software programs. Based on statistical studies of splicing signals, open reading frames, 6-mer, and length distributions of intron/exon sequences, an interactive computer program was developed.

This program is used routinely on newly sequenced genes in David Beach's laboratory. The early results from this program are encouraging; for example, among 19 introns of 9 new genes, 18 were predicted correctly and confirmed by the cDNA sequences. The one that was missed is a 39-bp in-frame intron. Without information about promoter/poly(A) signals, it was surprising to us that all of the predicted translational initiators and stops also seemed to be correct, except one where the initiation methionine codon, ATG, was disrupted by an intron after the single residue A!

We plan to continue the second subproblem by making more statistical studies on promoters, poly(A) signals, codon usage preferences, etc., in order to improve the interactive gene finder tools. To prepare for large-scale sequencing of the *Schizosaccharomyces pombe* chromosome, we plan to automate these tools and test the success rate against other programs. Eventually, these tools will be used to analyze other organisms (fly, mouse, human, etc.).

Collaboration with David Beach's Laboratory Yielded a 13-kb Resolution Physical Map of the 14-Mb Fission Yeast Genome

W.I. Chang, D. Lombardi, T. Marr

In collaboration with the Beach laboratory, we have completed both the development of a contig assembly algorithm and its application to the fission yeast *S. pombe* genome. More than 1000 sites have been determined and precisely ordered along the 14-Mb genome. A gene can now be localized to a 13-kb region (on average) with a simple hybridization experiment.

Our project is the first successful application of a nonrandom STS content detection method in mapping an entire genome. The novelty of our strategy is in the use of unique probes made by PCR from both ends of cosmid clones, selected from the library on the basis of "sample without replacement;" i.e., only clones that show no positive hybridization to existing probes are selected and made into probes for subsequent experiments. Two logical steps are carried out in tandem: Cosmid clones are assembled into contigs based on hybridization, and contigs are anchored onto chromosomes by integration with genetic and *Not*I/*Sfi*I restriction maps. We will describe the assembly process in greater detail.

Because our data are of high quality (7% false negatives, 2% false positives), the internal consistency of a contig is a powerful indicator of its correctness. On the other hand, inconsistencies suggest errors either in the hybridization data or in the putative ordering of probes. A given probe order gives rise to a set of inconsistencies that can be scored; a backtracking program analyzes all possible probe orders and finds the one that is most consistent with the data. Furthermore, any remaining inconsistencies become hypotheses of experimental error, which can be checked by consulting the original autoradiographs or running new experiments. Repetitive probes are rare (library was filtered for known repeats) and are detected when two self-consistent contigs contain the same probe. This error checking and the confirmation of our partial results allow us to proceed at a more aggressive pace, using a relatively sparse clone library (1920 cosmid clones representing five times coverage). The cosmid map was essentially complete after 1 year (7/91–6/92).

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GENOME SEQUENCE ANALYSIS

W.R. McCombie S. Matsumoto

The past two decades have been marked by an explosion in our knowledge of the structure and function of individual genes. One major factor in these advances has been the determination of the structure of a number of genes or their respective cDNAs at the level of their DNA sequence. Individual genes are the basic components of genetic information, but the most complex level of genetic organization is the genome

itself. Recent technical advances now make it feasible to consider the analysis of the structure and function of complete eukaryotic genomes at the level of their DNA sequence. This is the major effort of our laboratory. Our primary goal is to use the latest advances in automated DNA sequencing to analyze the genome of the fission yeast *Schizosaccharomyces pombe*. The groups of David Beach and Tom Marr began the *S. pombe* genome project by mapping the *S. pombe* genome and generating an ordered set of cosmid clones that comprise most of the genome. With this outstanding starting point, we will begin dissecting the *S. pombe* genome in collaboration with these groups. Three areas of research will be developed to accomplish this goal: (1) technology development to reduce the cost, increase the speed, and scale up the process of DNA sequencing; (2) sequencing a large number of cDNAs from *S. pombe*; and (3) sequencing large regions of the *S. pombe* genome. Since coming to the Laboratory in September of 1992, we have begun work on the first two of these areas.

Sequencing Technology Development

W.R. McCombie

Theoretically, to sequence large genomes efficiently, the most desirable strategy is based on a process of taking sequential sequencing steps with custom primers known as primer walking. This approach is not currently used in most large-scale projects because of the added cost of primers and the added time required and project management burden caused by the sequential nature of primer walking. This is opposed to the parallel scale up possible with random or shotgun approaches. Recently, Bill Studier (Brookhaven National Laboratory) demonstrated that in the presence of single-stranded binding (SSB) protein, three adjacent hexamers could prime sequencing reactions rather than a single 18 mer or longer primer (*Science 258:* 1787 [1992]). This advance offers tremendous potential to improve DNA sequencing efficiency. The entire set of 4096 hexamers could be readily synthesized in advance, thus eliminating the wait required for a new primer to be synthesized after each step in the sequencing. In addition, the cost benefits of being able to use multiple aliquots of a hexamer to prime many reactions rather than synthesizing a new 18 mer for each reaction are very significant. To make this approach applicable to large-scale sequencing, I have been collaborating with Bill Studier to adapt this hexamer-based reaction to current automated fluorescent DNA sequencers. This will eliminate the time-consuming, error-prone, and expensive step of manually reading sequencing gels and allow computer-readable sequences to be derived from hexamer-primed reactions. I have been carrying out experiments to adapt hexamer-primed reactions to both the Applied Biosystems (ABI) and Pharmacia DNA sequencers.

Hexamer-primed reactions require an initial extension of the hexamers at 0°C, followed by termination of the reactions at 37°C. With the Pharmacia chemistry, the most straightforward time to add fluorescent label to the extension products is during this initial extension reaction. The low temperature, however, reduces the incorporation of fluorescein-dATP to undetectable levels under the initial conditions attempted. The low temperature greatly reduces the incorporation of fluorescein-dUTP as well, but enough of this label is incorporated to allow detection and accurate sequence determination with control templates and standard sequencing primers. In addition, initial results indicate that the presence of single-stranded DNA-binding protein in the sequencing reactions interferes with fluorescent detection of the sequencing ladders.

The reactions with the Applied Biosystems instrument use fluorescent dideoxynucleotide analogs for termination and labeling. It should be possible to do these termination reactions at 37°C, following a 0°C extension with standard dNTPs to extend the hexamers enough to stabilize their binding to the template. This is potentially an advantage of the ABI system for hexamer-primed reactions, since the base analogs do not need to be incorporated at 0. Although this is not the way the ABI reactions are typically carried out, I have developed conditions to accomplish this. The presence of SSB, however, interferes with detection on the ABI machine as well as the Pharmacia. I am currently optimizing reactions primed with hexamers in which the SSB is removed by phenol extraction. I am also exploring alternate, less time-consuming ways to remove SSB.

Initial reactions on the Pharmacia gave 60 bases of sequence using adjacent hexamers, versus 400–500 with standard primers. Under the best conditions developed thus far, using the ABI machine, hexamer-

TABLE 1 Similarities of *S. pombe* cDNA Tags to Known Genes

Database identifier	Name
SP:RL5_YEAST	60S RIBOSOMAL PROTEIN L5 (YL3). >PIR:S
SP:UCR9_YEAST	UBIQUINOL-CYTOCHROME C REDUCTASE SUBUNI
PIR:S16810	*Ribosomal protein L7a.e - Yeast (Saccha
SP:RS12_RAT	40S RIBOSOMAL PROTEIN S12. >PIR:R3RT12
SP:TRXB_ECOLI	THIOREDOXIN REDUCTASE. >PIR:RDECT Thio
SP:ARD1_YEAST	ARREST-DEFECTIVE PROTEIN 1. >PIR:TWBYA
GP:MUSADIPCT_1	Mouse 19.5 kD protein mRNA, complete c
SP:EF1B_HUMAN	ELONGATION FACTOR 1-BETA (EF-1-BETA)
BB:B111730	ribosomal protein S13 [Schizosaccharom
SP:TRPD_METTH	ANTHRANILATE PHOSPHORIBOSYLTRANSFERASE
SP:TPIS_SCHPO	TRIOSEPHOSPHATE ISOMERASE (TIM). >PIR
SP:GBLP_HUMAN	GUANINE NUCLEOTIDE-BINDING PROTEIN BETA
GP:YSCRPS28A_1	ribosomal protein S28 [Saccharomyces c
SP:ILV5_YEAST	KETOL-ACID REDUCTOISOMERASE (ACETOHYDRO
SP:RS6_SCHPO	40S RIBOSOMAL PROTEIN S6. >PIR:R3ZP6E Ri
SP:RLD4_SCHPO	60S RIBOSOMAL PROTEIN KD4. >PIR:R5ZPD4

Sequences of cDNAs from *S. pombe* were used to search protein sequence databases using the Blast server of the National Center for Biotechnology Information at the National Library of Medicine. The program used for the search was Blastx, which translates DNA sequences into peptide sequences and searches protein databases for similarities. Those matches above a cutoff score of 95 are shown in the table above.

primed reactions read 350–450 bases but have substantially more ambiguities than standard reactions. I am continuing to optimize these reactions.

S. pombe cDNA Analysis

S. Matsumoto, W.R. McCombie

To aid in the analysis of genomic sequence, it is extremely useful to have available even partial sequences of as many cDNAs from that organism as is possible. The partial sequencing of cDNAs is also a rapid and inexpensive way to identify a large number of genes from an organism. In addition, since we will also be sequencing large genomic regions of *S. pombe*, we will be able to compare the genomic and cDNA approaches if we also undertake a cDNA-sequencing project. We have begun to partially sequence randomly selected clones from an *S. pombe* cDNA library (provided by Gregory Hannon). The translated sequences were then compared to a series of protein databases maintained by the National Center for Biotechnology Information. These include a translated version of GenBank. An initial analysis of about 50 of these sequences reveals that 35% of them are similar to previously known genes from other species or, in a few cases, genes previously identified in *S. pombe*. A list of these genes is shown in Table 1. We are currently working with Diane Lombardi and Tom Marr to develop a Sybase database for storage and analysis of these sequences. We are continuing to sequence cDNAs and to identify new potential homologs in *S. pombe* of interesting genes from other species. Our long-term goal is to sequence 10,000–15,000 genes in this way. This should represent the majority of genes in *S. pombe*.

NEUROSCIENCE

This section includes seven laboratories with interests in the development and function of the brain, with a particular emphasis on the signal transduction processes underlying learning and memory. Ron Davis is studying the genes, molecules, and cells involved in learning in the fruit fly, *Drosophila*. Grigori Enikolopov studies the role of nitric oxide, calcium, and other messengers in gene transcription as related to synaptic plasticity, and in particular, long-term potentiation. His laboratory recently demonstrated that nitric oxide potentiates the effect of calcium on transcription of the early immediate gene, *fos*. This finding has important implications for signaling processes during learning. Alcino Silva arrived at Cold Spring Harbor Laboratory in 1992 after making a finding of enormous importance. Genetically mutant mice deficient in the enzyme calcium/calmodulin kinase II are impaired in learning as well as in long-term potentiation. This observation is key to understanding the relationship between calcium-induced phosphorylation, potentiation of transmission at synapses, and animal learning. The laboratory of Dan Marshak is interested in growth factors and signaling processes involved in neuronal growth and differentiation. This includes the growth factor, S100β, a factor with an intriguing relationship to Alzheimer's disease. Hiro Nawa also studies peptide factors involved in differentiation of neurons. Many diffusible factors determine the neurotransmitter phenotype of brain neurons. Dr. Nawa's laboratory has provided beautiful evidence that a factor named BDNF is responsible for causing neurons to express the neurotransmitters, neuropeptide Y and somatostatin. This induction of neurotransmitter expression is potentially an important method of regulating the plasticity of synapses and, presumably, learning and memory. Tim Tully's group uses genetics to probe for genes involved in learning in *Drosophila*. Their excellent work has identified a handful of new learning genes. Yi Zhong works at the heart of neuroscience—studying synaptic plasticity and the excitable properties of neurons. He has uncovered numerous and important alterations in these properties in neurons from *Drosphila* learning mutants. The research of the seven Neuroscience laboratories is detailed below.

NEURONAL GROWTH AND DIFFERENTIATION

D.R. Marshak C. Brecher A. Rossomando
N. Chester M. Vandenberg
L. Peña

This laboratory conducts research on the biochemical basis of neuronal growth and differentiation. Overall, the questions that we approach in our research involve the decisions of neuroblasts to cease proliferation and to subsequently elaborate neuritic processes prior to terminal differentiation. These questions thus involve understanding how signal transduction systems that control cell proliferation in the neuroblast are altered upon becoming postmitotic as well as learning which growth factor molecules control these

switches within cells. Specifically, we are interested in the action of a growth-stimulating protein, S100β, that is produced by astrocytes in the brain. The mechanism of action of S100β is compared to that of other growth factors, such as those in the family of neurotrophins or the family of heparin-binding (fibroblast) growth factors. The involvement of such neurotrophic factors in degenerative diseases, such as Alzheimer's disease, has prompted our interest in the role of such factors in neuropathological processes.

Mechanism of Action of S100β

L. Peña, D. Marshak [in collaboration with E. Azmitia, New York University]

During this year, we received a large grant from the National Institute on Aging to conduct studies on the mechanism of action of S100β, a polypeptide growth factor produced by glial cells in the brain. This is a multisite program grant that brings together anatomists, pharmacologists, pathologists, molecular biologists, and biochemists that work on S100β. Our laboratory discovered the function of this molecule several years ago and have more recently discovered its abnormal levels in Alzheimer's disease. Our work is central to understanding the potential role of this factor in the pathology of neurodegenerative disease.

BINDING OF S100β TO TARGET CELL MEMBRANES

Binding assays were performed on membranes isolated from embryonic chick telencephalons and on intact C6 glioma cell cultures. Many of the basic parameters were systematically investigated in binding assays using ^{125}I-labeled S100β, including a buffer system, divalent cation (Mg^{++} and Ca^{++}) concentration, incubation times, ligand concentration, and membrane concentration. In addition, incubations of radioiodinated S100β with intact glial cultures were assayed. Optimal parameters were determined, but no saturable binding was observed when ligand concentration was varied. These data suggest that S100β does not bind to the target cell membranes in a classic receptor-ligand manner.

UPTAKE OF RADIOIODINATED S100β

Since S100β does not bind in a saturable manner to cell membranes, it is possible that S100β is taken up by cells and is active either within the plasma membrane or in another intracellular compartment. Two routes of study have been pursued. First, pulse-chase experiments were designed using S100β followed by localization with immunogold labeling and electron microscopic detection. Such analysis could be profitable, but primary cultures of neurons were unable to withstand the rigorous treatments required for electron microscopy. Feasibility with other cell types is being tested. Second, in collaboration with E. Azmitia, injection of radioiodinated S100β into rat brains was done in the fimbria-fornix tract and in the Raphe nuclei. In the fimbria, specific uptake has been observed in granular neurons of the dentate gyrus of the hippocampus. In the Raphe, it appears that uptake in a subpopulation of serotonergic neurons occurs. However, no retrograde transport of the ligand was seen from temporal structures to mid-brain perikarya. These data suggest that the primary action of S100β is within neuronal cell bodies proximal to the glial cells that produce the growth factor.

ROLE OF SEROTONIN

Data from lesion studies in vivo in rats indicated a role for the neurotransmitter, serotonin, in the release of S100β by E. Azmitia and P. Whitaker-Azmitia. In addition, studies of cultured neurons of the Raphe nuclei show that S100β is a potent neurotrophic factor for these serotonergic neurons. Unfortunately, reagents for studying the serotonergic systems were not available as they are for catecholaminergic and cholinergic systems. Therefore, we set out to produce antibodies to two proteins that are critical to these studies: (1) tryptophan hydroxylase, the mixed function oxidase that is the rate-limiting step in serotonin biosynthesis, and (2) serotonin receptor type 1a, which has been linked pharmacologically to S100β induction and cAMP-mediated responses in glia. We chose to produce these antibodies in rabbits using antigens based on synthetic peptides that correspond to particular regions of the molecules. Regions chosen for antigenic sites are unique to these proteins and are thought to be located on the proteins' surfaces. High-titer antisera were thus prepared and are excellent tools for immunohistochemical studies as well as biochemical analysis of proteins.

Distribution of S100β in Human Brain Diseases

D. Marshak, M. Vandenberg [in collaboration with W.S.T. Griffin, University of Arkansas, and P. Whitaker-Azmitia, SUNY Stony Brook]

The proximity of S100β-containing astrocytes to neuritic, amyloid plaques in the brains of Alzheimer's disease (AD) patients suggests a functional role for S100β in the pathology of the disease. Gliosis and elevated S100β accompanies several neurodegenerative diseases, and the elucidation of the role of this growth factor in degeneration is important to our understanding of the etiology of the diseases. In addition, basic research in glial-neuronal interactions will help us to learn the fundamental processes that control brain development and adult function. In collaboration with W.S.T. Griffin, radioimmunoassays for S100β are being done on a variety of brain regions from autopsy tissue of AD patients compared to age-matched controls. Temporal lobe regions that are clearly affected by amyloid deposition and degeneration have elevated S100β in reactive glia. In a separate study, samples from patients suffering from AIDS dementia were observed to have increased S100β but not to as high a level as in AD. Down's syndrome brain tissue has been analyzed in collaboration with P. Whitaker-Azmitia to analyze the levels of S100β in infants. There appears to be elevation, even in the absence of amyloid deposition. In another study, patients with a ring chromosome 21 that have some clinical symptoms were shown to have only two copies of the S100β gene, unlike full trisomy 21 patients, who have three copies.

In Alzheimer's disease and Down's syndrome, severely afflicted brain regions exhibit up to 20-fold higher protein levels of S100β, and astrocytes surrounding neuritic plaques exhibit higher protein levels of S100β. A major constituent of the plaques is β-amyloid protein, which has been reported to have both neurotrophic and neurotoxic effects in vitro. We examined the responses of central nervous system glia to a β-amyloid peptide. Primary astrocyte cultures, obtained from neonatal rats, and rat C6 glioma cells were synchronized by serum deprivation and treated with βA(1-40), a synthetic fragment of β-amyloid. A weak mitogenic activity was observed, as measured by [^{3}H]thymidine incorporation. Northern blot analysis revealed increases in S100β mRNA within 24 hours, in a dose-dependent manner. Figure 1 shows an experiment that indicates the increase in S100β mRNA in C6 cells treated with β-amyloid. Nuclear runoff transcription assays showed that βA(1-40) specificially induced new synthesis of S100β mRNA in cells maintained in serum but caused a general elevation of several mRNA species in cells maintained under serum-free conditions. At the protein level, corresponding increases in S100β protein synthesis were observed in response to the βA(1-40) peptide, measured by immunoprecipitation of ^{35}S-labeled cellular proteins. The data indicate that S100β expression can be influenced directly by β-amyloid.

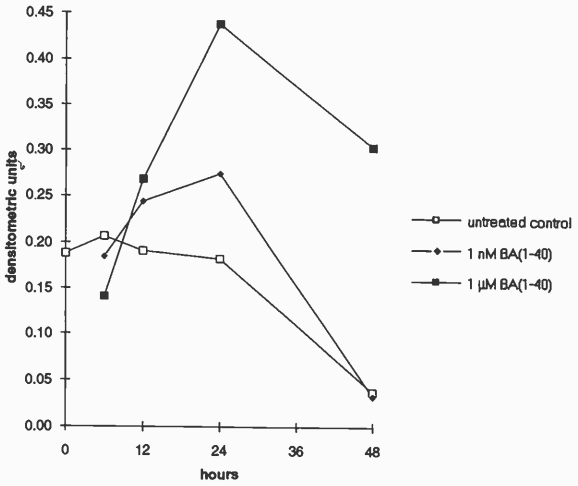

FIGURE 1 Experiment showing increase in S100β mRNA in C6 cells treated with β-amyloid.

Protein Kinase Modulation during Neuronal Differentiation

A. Rossomando, L. Peña, D. Marshak [in collaboration with G. Landreth, Case Western Reserve, Cleveland, Ohio]

We have continued our studies of protein kinase responses to growth factors in neuronal cells. Two model systems are currently in use: chicken embryo neurons stimulated with S100β and rat pheochromocytoma (PC12) cells stimulated with nerve growth factor (NGF) or epidermal growth factor (EGF). In collaboration with Gary Landreth in Cleveland, we identified a new protein kinase that responds to NGF and EGF in PC12 cells and that phosphorylates the c-

fos proto-oncogene product. This is of significance because it suggests that neuronal responses of increased c-*fos* expression might be regulated by phosphorylation of the protein product.

A complete study of $p34^{cdc2}$ kinase and MAP kinases in PC12 cells that respond to NGF has been conducted by A. Rossomando. The rat pheochromocytoma cell line, PC12, undergoes morphological and biochemical differentiation into sympathetic neurons in culture under the influence of NGF. The enzyme $p34^{cdc2}$ kinase, which is critical to the induction of mitosis, appears to be down-regulated during NGF-stimulated differentiation. The previously identified p46 protein seems to be a form of MAP kinase in these cells. This protein kinase is known to be stimulated indirectly by growth factor receptor tyrosine kinases and associated molecules. We are currently working on the interactions between this family of kinases and the cell-division-cycle-regulated kinases, such as $p34^{cdc2}$. These studies will enable us to work out pathways of signaling from the surface of the neuron to the genome.

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MOLECULAR AND CELLULAR BIOLOGY OF LEARNING

R. Davis G. Bolwig, J. Bonacum, J. Cherry, C. Chromey, J. Crittenden, B. Dauwalder, J. DeZazzo, K. Han, S. Hespelt, M. Joyce, A. Nighorn, E. Skoulakis, R. West, K. Wu

Our laboratory continues with its long-term goals of probing the molecular and cellular biology of learning. We approach these goals using the techniques of genetics, molecular biology, biochemistry, behavior, and anatomy. In general, the techniques of genetics and behavior are used to identify genes required for normal learning/memory, the techniques of molecular biology and biochemistry are used to clone the required genes and to characterize the gene products, and anatomical methods are used to trace the flow of

information in the brain and to understand where the genes are required to be expressed for normal learning/memory.

A major and important story contributed by our laboratory in 1992 was the characterization of the rutabaga gene of *Drosophila*. With molecular and behavioral techniques, we demonstrated that rutabaga encodes a calcium/calmodulin-stimulatable adenylyl cyclase and that this gene is preferentially expressed in neural centers called mushroom bodies. These structures are thought to mediate olfactory learning in insects. Progress on other projects in the laboratory is described below.

The laboratory expanded in 1992. During the past year, we have welcomed Jim Bonacum, Marianne Joyce, Sue Hespelt, Jim DeZazzo, Gert Bolwig, Jill Crittenden, Kay Han, and visiting scientist Bob West into our laboratory. We said goodbye to our friend and colleague, Pyung-Lim Han. Han finished an excellent thesis on the rutabaga locus and initated postdoctoral work at the Baylor College of Medicine.

Drosophila Protein Kinase A

E. Skoulakis [in collaboration with D. Kalderon, Columbia University]

The fly stock, MB581, was isolated in an enhancer-detector screen to search for mushroom body genes and was shown to contain a transposable element in the unique gene for the catalytic subunit (*dc0*) of protein kinase A (PKA). In situ hybridization to tissue sections and immmunohistochemical analysis demonstrated that *dc0* is preferentially expressed in the mushroom bodies. Thus, the preferential expression of PKA in the mushroom bodies along with the rutabaga-encoded adenylyl cyclase (AC) and the dunce-encoded cAMP phosphodiesterase (PDE) lends strong support for a role of the cAMP cascade in these neurons to serve learning/memory processes.

Molecular characterization revealed that the transposon in MB581 was inserted in exon 1, 71 bp downstream from the transcriptional start site of *dc0*, thus disrupting the gene. This disruption reduces the amount of *dc0* RNA to barely detectable levels as assayed by in situ hybridization with homozygous mutant embryos. The insertion produces recessive lethality and a 35–40% reduction in PKA activity in heterozygous adults due to the reduction in the level of the DC0 protein. A similar decrease in activity and a lethal phenotype are exhibited by a different hypomorphic allele of this gene (B10) isolated in an screen by ethylmethanesulfonate (EMS) mutagenesis (D. Kalderon, unpubl.). The reduction in PKA activity in B10 is likely the effect of a missense mutation on the structure of the protein and/or activity of DC0. Surprisingly, heteroallelic MB581/B10 animals can be obtained that appear to be normal externally and normal with respect to brain structure. However, they exhibit only 20–25% of the PKA activity of control strains.

The effect of reduced PKA activity on behavior was investigated by determination of the learning/memory phenotype of MB581 and B10 heterozygotes and the MB581/B10 heteroallelic animals using an olfactory classical conditioning paradigm. Initial learning of MB581 is slightly reduced compared with the control strains and B10 heterozygotes. Interestingly, memory assayed 30 minutes or later is normal. Similarly, B10 heterozygous animals exhibit a slight dominant effect on learning. Thus, the 35–40% reduction in PKA activity appears to have a mild effect on learning but is not limiting for memory. The MB581/B10 heteroallelic animals exhibit a 50% reduction in both initial learning and memory. This behavioral effect is not due to the inability of the mutants to perceive the stimuli demonstrated by control experiments in which both heterozygotes and the heteroallelic animals are indistinguishable from the control strains. These results demonstrate unambiguously that PKA plays a cardinal role in learning and memory processes mediated by the mushroom bodies.

Rutabaga Structure and Expression

P.L. Han [in collaboration with L. Levin and R. Reed, Johns Hopkins School of Medicine]

Several of the lines identified by enhancer detection with preferred expression of β-galactosidase in mushroom bodies were shown to have a *P*-element insertion at the cytogenetic position corresponding to the rutabaga locus, the second best characterized learning gene of *Drosophila*. To determine whether these insertion elements had indeed landed in the rutabaga locus, the genomic DNA flanking the insertions was isolated and compared to that isolated from

an adenylyl cyclase (AC) gene thought to be the rutabaga function. Seven insertions were identified within 2 kb upstream of the transcription start of the AC gene. In addition, we were able to show that several of these insertions were new alleles at the rutabaga locus behaviorally. By RNA in situ hybridization with AC probes and immunohistochemistry with anti-AC antibodies, we demonstrated that the AC RNA is preferentially expressed in mushroom bodies, that the AC immunoreactivity was concentrated in mushroom body neuropil, and that the insertion elements of the new *rutabaga* alleles compromised the expression of the AC gene. These observations add great strength to the idea that mushroom bodies are neural centers for insect learning and that the products of known learning mutants have their effects by altering mushroom body cell physiology.

Structure and Function of Dunce

Y. Qiu

The dunce locus presented a special challenge in identifying its transcriptional units and identifying the biological function of each. The gene extends over more than 148 kb. This makes dunce the largest *Drosophila* locus characterized to date. Transcription of dunce begins at a minimum of five sites from standard molecular analysis by primer-extension and S1-nuclease experiments. Because of the complexity of the transcriptional units, we initiated a series of experiments to define the biological role of each.

A series of deficiency chromosomes known to have breakpoints in and around dunce were analyzed by Southern blot analysis. This allowed for the identification of deficiencies that remove transcription start site (tss) 1 and 2, tss1-3, tss1-4, and all known transcription start sites. These flies were assayed for normal cAMP phosphodiesterase (PDE) activity, the known product of dunce, for elevated mushroom body expression of the PDE, for female fertility, since *dunce* mutations cause female sterility, and for learning/memory activity. Surprisingly, removal of tss1 and tss2 has no detectable effect on any of the phenotypes. tss3 was required for elevated mushroom body expression but not for female fertility or initial learning. tss4 contributed to learning and the female fertility function and tss5 contributed to female fertility. The results suggest that the structural complexity of the gene is needed for serving unique biological functions.

Cell Adhesion Molecules and Learning

K. Wu [in collaboration with C. Goodman, University of California, Berkeley]

Among the enhancer-detector lines, one line named MB2225 has its *P*-element insertion site cytogenetically at 4AB. This is a locus housing the gene for a *Drosophila* cell adhesion molecule, fasciclin II (fas II), which is originally identified as a molecule involved in the early development of the nervous system. When tested for their ability to associate an odor with electric shock, flies from this line showed defective association and memory at all time points tested up to 3 hours after training.

To clarify the relationship between fas II and MB2225, the genomic fragment next to the *P*-element insertion site in MB2225 was obtained by plasmid rescue. Using this fragment for Southern hybridization and DNA sequence analysis, the insertion site in MB2225 was mapped to the first exon of the fas II gene at a position between the transcription start site and the open reading frame. Therefore, MB2225 was expected to be a fas II mutant. To confirm this, protein expression was analyzed by immunohistochemistry using antibodies raised against the transmembrane form of the Fas II protein. In head sections of wild-type adult flies, the protein is expressed mainly in the mushroom bodies and the ellipsoid body of the central complex. This expression is missing in MB2225. Because of the hypothetical role for mushroom bodies in learning, Fas II expression in these structures is consistent with the possibility that Fas II functions in adult behavior in addition to its developmental functions.

lightminded, a Putative New Learning and Memory Mutant

B. Dauwalder, J. Crittenden

Enhancer-detector line 2063 shows preferential expression of β-galactosidase in the mushroom bodies. Flies homozygous for the *P*-element insert are defi-

cient in learning and memory in a classical negatively reinforced learning paradigm. Initial learning as well as retention over the 180 minutes tested is lower than that of wild type. Control experiments confirmed that mutant flies perceive the stimuli used for conditioning (olfactory cues and electrical shock) in a manner indistinguishable from that of wild type, suggesting a defect specific for the learning/memory process. Another *P*-element line with an insert only 200 bp from 2063 has also been shown to be deficient in learning/early memory, and this may be allelic to the first.

We have isolated genomic DNA flanking the *P*-element insertion site and used it to screen a head-specific cDNA library. Two classes of transcripts have been identified. Several head-specific RNAs were detected by the cDNA probes, some of which are absent or strongly reduced in the mutant. Future work will include characterizing these sequences and their spatial expression pattern.

Mushroom Body Lines

C. Chromey, S. Hespelt

Beginning with the 90 mushroom body stocks that were previously isolated, approximately 65 have been tested in an olfactory, classical learning situation to search for learning/memory deficits. The preliminary results have been intriguing; several new putative mutants have been uncovered with differential effects on the shape of the memory curve. Five distinct classes of memory curves have been observed to date: (1) stocks that score lower at all time points tested, (2) stocks that score low at early time points but normal at later ones, (3) stocks with normal scores immediately after training but with a rapid memory loss, (4) stocks that score lower than normal at early times but higher than normal at late times, and (5) stocks with normal scores immediately after training but with enhanced scores later. These classes may represent the beginning of the genetic dissection of different phases of memory.

Mushroom Body Cell Culture

K. Han

The primary goals of this project are to identify the neurotransmitter receptor(s) coupled to adenylyl cyclase and to identify the neurotransmitter(s) expressed by mushroom body cells for communication with follower neurons. An enhancer-detector line that expresses β-galactosidase exclusively in mushroom body cells was used. Third instar larval brains were dissociated enzymatically and mechanically and plated; 20,000–50,000 cells are recovered from each brain. Staining of fixed cells with X-gal after 1 day of culture or immediately after dissection revealed that approximately 2% of total cells expressed *Escherichia coli* β-galactosidase. This proportion is comparable with the estimated number of mushroom body cells in the brain, suggesting no selection for or against mushroom body cells in culture. The cells were also identified after hypotonic shock with FDG, a fluorescent substrate for β-galactosidase. Thus, mushroom body cells can be identified among heterogeneous cultures of brain neurons. The development of this procedure will allow the electrophysiological analysis of mushroom body cells as well as sorting the cells for further biochemical or cell biological experiments.

Mammalian Homologs of Dunce

J. Cherry, J. Bonacum

The study of *Drosophila* has yielded much of our current understanding of the cellular mechanisms underlying behavior. A number of genes that appear to be critical for the processes of learning and memory formation have been identified in this species, including the dunce gene, which encodes a cAMP-specific phosphodiesterase. We are now in a position to ask whether these genes are structurally and functionally conserved in other species. We have thus begun to examine the mammalian homologs of the *Drosophila* dunce gene. Four distinct dunce homologs have so far been reported in mammals; we used polymerase chain reaction (PCR) to partially clone the mouse dunce homologs from rat clones obtained from M. Wigler's laboratory. Antibodies have been generated to each of the mouse dunce gene products, and we are now in the process of determining specifically where in the brain the Dunce proteins occur. Similarly, antisense RNA probes to brain tissue sections should indicate where each gene is expressed. Our hope is that one or more of the mouse dunce genes will be

found in regions of the brain associated with learning and memory, such as the hippocampus and cortex. Ultimately, the question of function of the dunce genes in mice will be addressed by gene knockouts using homologous recombination in embryonic stem cells.

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GENETICS OF LEARNING AND MEMORY IN *DROSOPHILA*

T. Tully R. Mihalek, C. Jones, M. Regulski, D. Wood, G. Hannon, M. Del Vecchio, S. Boynton, C. Brandes, F. Imrie (URP), K. Han, T. Preat

Our first full year at Cold Spring Harbor Laboratory has been a very productive one. Genetic analyses of genes involved with learning and memory in fruit flies have moved in iterative fashion from one phenotypic level to another, yielding important biological information about several loci. In the last year, we have continued to characterize our newly isolated learning/memory genes—*latheo* and *linotte*—at molecular, anatomical, developmental, and behavioral levels of analysis. Work on *latheo*, in particular, suggests that this is quite an interesting gene. We have sequenced our newly isolated temperature-sensitive allele of *pale*, revealing a single-amino-acid substitution in the tyrosine-hydroxylase coding sequence. We also have initiated two "reverse genetic" studies on the RI regulatory subunit of protein kinase A (PKA) and on *Drosophila* nitric oxide synthase (NOS) homologs. Finally, we continue our behavioral analyses of normal and mutant fruit flies. We have demonstrated additional vertebrate-like properties of learning and memory, including inhibitory conditioning, US pre-exposure, and formation of a bona fide long-term memory (LTM). Initial results from the latter experiments suggest that LTM does not form in *amnesiac* mutants.

"Forward Genetics" of *latheo*, *linotte*, and *pale*

T. Tully, S. Boynton, R. Mihalek, C. Jones, D. Wood, G. Hannon, F. Imrie, K. Han [in collaboration with Y. Zhong, Cold Spring Harbor Laboratory]

Last year, we summarized the basic behavior-genetic characterization of *latheo*, a new gene involved with associative learning/memory identified while at Brandeis University. Along with the development of behavioral control experiments to verify that *latheo* mutants sensed, and responded to, odors and electric shock normally, we also generated lethal alleles of the gene upon excision of the *P*-element mutator.

This year, we have looked more closely at the developmental lethal phase in several genetic variants of *latheo*. Lethal *latheo* mutants hatch, grow through the larval instars, and pupate normally, but no flies eclose as adults. This result prompted us to look inside third instar larvae just before they pupated. Interestingly, we could not find any imaginal discs, and central brain structures—particularly optic lobes—appeared to be small. Moreover, we failed to detect any mitotic activity in the larval central nervous sys-

tem after a 1.5-hour pulse of bromodeoxyuridine, which labels replicating DNA during cell division. This overall developmental syndrome is intriguing, especially since adult brains of flies homozygous for viable *latheo* alleles have no obvious structural defects (Boynton and Tully 1992). Thus, *latheo* may be required for normal development of adult structures. Furthermore, our colleague here at the laboratory, Dr. Y. Zhong (see his report in this section), has discovered a functional defect at the neuromuscular junction in viable *latheo* larvae, suggesting that the *latheo* gene is also required for normal synaptic function—at least in motor neurons.

We began molecular cloning of *latheo* by probing a phage library of genomic DNA fragments from *latheo*^{P1} flies with the labeled *P*-element sequence (the *latheo* *P*-element was not a newer "plasmid-rescuable" version). Eleven positives were identified from a total of 270,000 plaques. We knew from restriction analysis of *latheo* genomic DNA that a *Hin*dIII digest of the correct phage would yield a 700-bp fragment containing 40 bp of *P*-element DNA and 660 bp of "flanking" genomic DNA. Three of the 11 phage clones yielded such a *Hin*dIII fragment. This fragment then was gel-purified and subcloned into a plasmid. The 700-bp *Hin*dIII fragment then was used to probe a phage library containing genomic DNA from wild-type Canton-S flies. Three clones were identified; subsequent restriction analysis characterized 24.2 kb of genomic DNA of the *latheo* region. In particular, a 1.9-kb *Eco*RI-*Hin*dIII fragment spanned the *P*-element insertion site, with 200 bp to the left and 1.7 kb to the right. This 1.9-kb *Eco*RI fragment was selected for screening cDNA libraries. Three partial cDNA clones were identified from a Canton-S adult head-specific library, with the largest clone containing a 3.2-kb insert. This larger cDNA clone and the 1.9-kb *Eco*RI-*Hin*dIII genomic fragment both identify 3.9-kb and 2.6-kb alternatively spliced messages on a Northern blot containing poly(A)$^+$ RNA from wild-type Canton-S flies. Importantly, a 3.6-kb (truncated?) RNA message is present in *latheo* RNA, and the amount of 2.6-kb message is reduced (quantified by overprobing Canton-S and *latheo* RNAs with myosin light chain 1; Fig. 1). We currently are sequencing this putative *latheo* cDNA.

Molecular cloning of *linotte* also is proceeding quickly. The *linotte* *P*-element was a newer "plasmid rescuable" version, so identifying genomic DNA flanking the *P*-element insert was more straightforward. *linotte* genomic DNA was cut with *Sac*II, ligated at low concentration, and transformed into DH5Alpha *E. coli*, and clones were selected for ampicillin resistance. We obtained four colonies. Restriction analysis of these colonies revealed some fragments from *PlacW* and a *Hin*dIII-*Sac*II fragment of "flanking" DNA. We used this fragment to probe a Canton-S genomic library. Four genomic phage clones were identified, and subsequent restriction analysis yielded a 35.8-kb genomic map of the *linotte* region. We probed the Canton-S adult head-specific cDNA library with a 5.7-kb *Hin*dIII genomic fragment near the *P*-element insertion site. Four cDNA clones were isolated, falling into two sets that did not cross-hybridize. We have shown that the set of cDNAs farthest away from the *P* insert corresponds to a previously cloned gene. We probed a Northern blot containing poly(A)$^+$ RNA from wild-type and *linotte* adults with a 3.0-kb cDNA clone from the more proximal set. This cDNA hybridized to one 3.5-kb message in wild-type and mutant flies. When hybridization intensities were corrected for different amounts of RNA in each lane (quantified with a probe for myosin light chain 1), the *linotte* message appeared to be sixfold less abundant than normal. Thus, we presume to have a partial cDNA clone representing a putative *linotte* adult message. Addi-

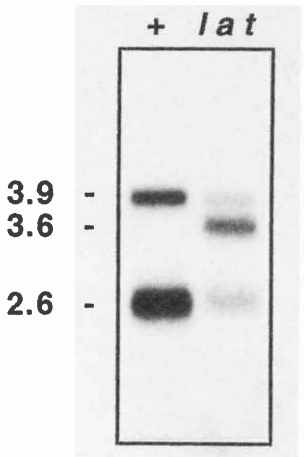

FIGURE 1 Northern blot analysis of poly(A)$^+$ RNA from wild-type Canton-S and *latheo* flies probed with a 1.9-kb *Eco*RI-*Hin*dIII genomic fragment spanning the *latheo* *P*-element insertion site. This genomic fragment (and a 2.6-kb cDNA clone from a Canton-S adult head-specific library) hybridizes in wild-type flies to 3.9- and 2.6-kb messages, the levels of which are reduced in mutant flies (relative amounts of RNA in each lane were quantified by overprobing with sequence to myosin light chain 1). An additional 3.6-kb message also is apparent in *latheo* flies, suggesting the presence of an aberrant message.

tional cDNA clones have been identified, and the entire coding region has been sequenced, revealing no homologies with known genes. We currently are attempting phenotypic rescue of *linotte*'s learning defect with germ-line transformants carrying the putative *linotte*$^{+}$ message.

The *linotte P*-element is an "enhancer-trap" construct containing a β-galactosidase (β-gal) "reporter" gene behind a weak promoter. Consequently, endogenous enhancer elements near the *P*-element insert can drive expression of the β-gal gene. We have examined frozen tissue sections of *linotte* adult brains and whole mounts of *linotte* third instar central nervous systems for β-gal activity. The latter show a distinct and strong pattern of expression primarily in the lateral hemispheres of the brain lobes. In addition, two (clusters of) neurons near the anterior end of the midline and a few neurons scattered throughout the medial brain hemispheres show some β-gal activity. Little activity is apparent in the ventral ganglia (Fig. 2). Frontal sections of adult heads show β-gal activity in the optic lobes, in a cluster of cells anterior and medial to—but not in—the mushroom body calyces, in two cells (or clusters) ventral and lateral in the subesophageal ganglion, and in scattered neurons throughout the brain. Sagittal sections of fly bodies revealed no significant β-gal activity elsewhere.

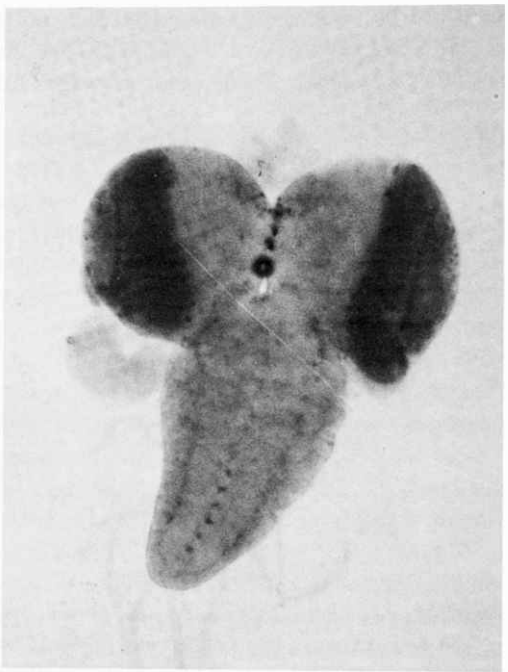

FIGURE 2 Enhancer-driven β-gal activity in a whole-mount larval third instar central nervous system of *linotte* mutants. Strong expression is seen in the lateral hemispheres of the brain lobes, primary anatomical sites of developing optic lobes. Some staining also is apparent in two clusters of neurons near the anterior end of the midline of, and in a few other neurons scattered throughout the medial brain hemispheres.

The prominent β-gal activity in optic lobes of larvae and adults was surprising to us, since *linotte* mutants were identified by disruption of olfactory learning, which itself does not require optic lobe function (T. Tully, unpubl.). Thus, we decided to assay phototactic behavior in *linotte* mutants. Again to our surprise, we discovered that phototactic behavior was perfectly normal in mutant flies. These results suggest that the enhancer elements driving expression of β-gal may not regulate the *linotte* olfactory learning/memory gene. Instead, the enhancers may regulate a different (unknown) gene.

Finally, we have finished sequencing the coding region of our temperature-sensitive allele of *pale*, the structural gene for tyrosine hydroxylase. By comparing this sequence to the sequence from "parental" nonmutagenized flies, we have identified a single C-T nucleotide substitution in position one of codon 415, changing an asparginine to a cysteine. Behavioral experiments on *pale*^{ts} mutants unfortunately have not been as straightforward. Glyoxylic acid histofluorescence experiments on adult brain tissue sections have revealed that initial mutant deficits in catecholamine levels return to normal over several generations when mutant flies are maintained as homozygotes. This "accumulation of modifiers" phenomenon has been observed previously with other learning/memory mutants and can be controlled by continuous outcrossing of mutant stocks to wild-type Canton-S flies. This procedure "equilibrates" genetic backgrounds. We are in the process of outcrossing the *pale*^{ts} mutants before proceeding with behavioral experiments to determine if *pale*^{ts} mutants disrupt olfactory learning/memory.

"Reverse Genetics" of RI and NOS

T. Tully, M. Regulski, M. Del Vecchio [in collaboration with K. Kaiser, University of Glasgow]

Using a polymerase chain reaction (PCR) primer corresponding to the *P*-element sequence and one cor-

responding to the sequence from the RI regulatory subunit of protein kinase A (PKA), Dr. K. Kaiser has "directed" two independent *P*-element inserts near the gene, resulting in homozygous viable flies. Northern blot analysis has revealed larger RNA messages from both RI mutants, suggesting that the *P*-element inserts are in the coding sequence. We have shown that homozygotes of each mutant allele (*11D4* and *7I5*) perform abnormally in Pavlovian conditioning experiments (Fig. 3). These performance deficits of the RI mutants most likely result from disruption of associative learning per se, since olfactory acuity and shock reactivity in the mutants are normal (data not shown). Initial learning levels are low and subsequent memory decays are normal, suggesting that these RI mutations affect "learning" rather than "memory." To our knowledge, this is the first indication in any species that the RI regulatory subunit of PKA specifically may be involved with learning.

Nitric oxide (NO) is produced from arginine by nitric oxide synthase (NOS) and has emerged as an important messenger molecule involved in a wide variety of physiological processes in different organs and tissues. Macrophages synthesize NO for their bacteriocidal and tumoricidal actions. Endothelial cells of blood vessels send NO as a vasodilating factor. More recently, NO has been proposed to be a neuronal (retrograde) messenger during long-term

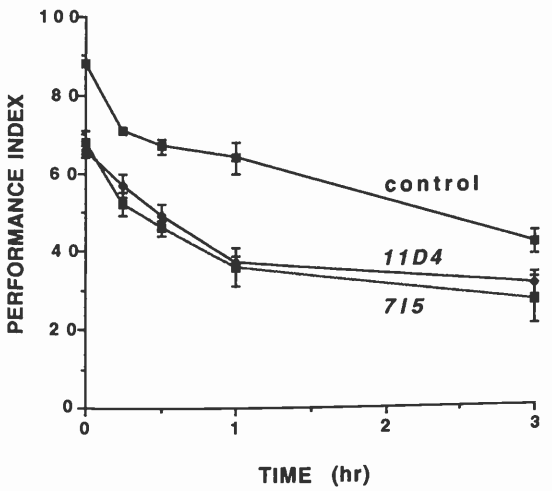

FIGURE 3 Memory retention in wild-type adult flies (control) and mutants homozygous for a *P*-element insert in the RI regulatory subunit of protein kinase A (PKA; *11D4* and *7I5*). A performance deficit is seen immediately after standard training with a Pavlovian olfactory conditioning procedure. Subsequent memory decay during the first 3 hr after training, however, appears normal in mutant flies.

potentiation, a neurophysiological phenomenon thought to underlie vertebrate learning and memory. The latter experiments convinced us to look for NOS homologs in flies, ultimately to mutate such *Drosophila* NOS homologs followed by behavioral analysis.

We used fragments of the rat-brain-specific NOS cDNA (kindly provided by Dr. S. Snyder) as heterologous probes to screen Southern blots of *Drosophila* genomic DNA. A 1.3-kb *Bgl*II rat cDNA fragment includes the FAD- and NADPH-binding domains, which we thought might be conserved between rat and fruit fly. At reduced stringency (43% formamide), this probe hybridized to eight to ten *Eco*RI fragments from Canton-S genomic DNA. We then used the rat cDNA fragment to screen a Canton-S genomic *Sau*3A phage library. Fifty of the strongest hybridization signals from 120,000 plaques were purified, and DNA was extracted, cut with *Eco*RI, electrophoresed, and blotted onto nitrocellulose. These blots were then probed with the 1.3-kb *Bgl*II rat cDNA fragment. Genomic fragments that hybridized with the rat probe were gel-purified and used to probe the same blots at high stringency. This approach allowed us to arrange the genomic phage clones into eight different sets. We also hybridized our gel-purified *Sau*3A fragments back to our Southern blots of *Eco*RI-cut Canton-S genomic DNA to verify that our clones still hybridized to the original fragments.

To date, we have worked most with phage set 1. The 1.3- and 0.6-kb *Bgl*II fragments of the rat cDNA hybridize to a 2.4-kb *Eco*RI fragment in set 1, whereas the 0.8-kb *Bgl*II fragment of the rat cDNA hybridizes to a 13-kb *Eco*RI fragment. These results suggested extensive homology with a rat NOS in this region of the fly genome. Consequently, we sequenced a 720-bp subclone of the 2.4-kb *Eco*RI fragment, revealing one uninterrupted open reading frame of 240 (deduced) amino acids. When compared to NOS proteins over the same region, this 720-bp fragment shows 48% identity to a cow endothelial NOS protein, 49% identity to a mouse macrophage NOS protein, and 45% identity to a rat brain NOS protein. This stretch of the fly protein also contains highly conserved FAD- and NADPH-binding domains, arranged in the same order as in other NOSs. A lesser homology (32% identity) was detected for cytochrome P450 reductase (CPR), a protein also related to other NOSs. (CPR shares extensive homology with the carboxy-terminal half of NOSs, most likely des-

cending from a common ancestor protein.) Thus, the 720-bp *Drosophila* sequence appears to be more closely related to other NOSs than to CPR. No other homologies were identified in protein database searches.

Given the sequence homology of our 2.4-kb *Eco*RI DNA fragment with other NOSs, we wanted to determine the spatial distribution of RNA for the corresponding fly gene. Strand-specific RNA probes were synthesized and hybridized in situ to frozen sagittal sections of adult flies. The antisense probe hybridized to trachea in the brain, to trachea around the flight muscles, and to a specific region of the gut. This pattern of expression is consistent with the notion that our 2.4-kb *Eco*RI fragment is part of a gene encoding the fly homolog of endothelium-specific NOS. We continue to characterize the other seven sets of genomic phage hopefully to identify a brain-specific NOS homolog.

Behavioral Analyses of Long-term Memory, Inhibitory Conditioning, and US Pre-exposure Effects

T. Tully, T. Preat, M. Del Vecchio [in collaboration with C. Sahley, Purdue University]

Years ago, we showed that after one "standard" training cycle of Pavlovian conditioning—in which flies were shocked in the presence of one odor but not in the presence of a second odor—memory retention of conditioned odor responses normally decayed to about 18% of initial values within 24 hours and to 5% of initial values within 4 days. More recently, we have automated our training apparatus and, consequently, have been able to demonstrate much longer-lasting memory after extended training. Ten training cycles with a deliberate rest interval between each ("spaced" training) produced memory that decayed only to 50%, 43%, and 27% of initial values within 1, 4, or 7 days. Interestingly, ten cycles of training without a rest interval between each cycle ("massed" training) produced moderate memory levels (33% of initial levels) within 24 hours but then decayed to 5% of initial levels within 4 days (as was the case for one cycle of training). Thus, spaced training was required to produce long-lasting memory, a property of memory formation ubiquitously observed in vertebrates.

Last year, we mentioned that third instar larvae could be trained to avoid a shock-paired odor and that they remembered this odor avoidance through metamorphosis. In these experiments, we also showed that such a stable long-term memory (LTM) was not apparent in *amnesiac* mutants. This result prompted us to look this year at LTM in *amnesiac* adults after extended training. We have discovered that spaced training of *amnesiac* flies produced a 1–4-day retention curve similar to that of wild-type flies after massed training (see above). These results suggest that *amnesiac* mutants are not able to form LTM. Further analyses with additional mutants are under way.

We continue to demonstrate various properties of learning and memory that are known to exist in most, if not all, vertebrates. In the past year, we have shown that flies become less repelled by an initially aversive odor if that odor is explicitly *unpaired* with electric shock, a phenomenon called inhibitory conditioning. Under such conditions, flies in essence learn that the odor is "safe."

We also have demonstrated reduced excitatory conditioning (which is an increase in avoidance when a stimulus is *paired* with shock) to odors after flies have been exposed to shock alone. In more general terms, this phenomenon is called the "US pre-exposure effect." Interestingly, nearly 100 years of behavioral work never has been able to resolve whether US pre-exposure effects are due to underlying associative or nonassociative processes. We decided to address the issue genetically, since *latheo* mutants show defective associative learning but normal habituation (nonassociative learning) and therefore have "genetically dissected" the two processes. We discovered that *latheo* mutants show perfectly normal US pre-exposure effects, which favors the argument that the phenomenon results from habituation to shock-alone cues. In this manner, we intend to continue our genetic dissection of learning and memory with *Drosophila* mutants.

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REGULATION OF NEUROTRANSMITTER DEVELOPMENT AND ITS IMPLICATION IN NEURAL PLASTICITY

H. Nawa Y. Bessho
K. Mizuno
M. Waga

Neurons communicate with each other using a large variety of neurotransmitters and neuropeptides. Despite this enormous diversity, each functional neural pathway can be recognized by the particular set of transmitters, neuropeptides, and receptors employed. Clearly, this "chemical coding" of pathways is critical for controlling neural function and behavior. The long-term objective of our laboratory is to define the molecular and cellular mechanisms that regulate neurotransmitter and neuropeptide phenotypes in the nervous system. In particular, we are interested in the physiological consequences of the regulation. The alteration in neurotransmitter/peptide levels presumably changes the mode of neurotransmission at each synapse and consequently modulates neural functions such as cognition and memory.

We have identified multiple neuronal differentiation factors in heart-cell-conditioned medium (CM), which control neurotransmitter/peptide expression in peripheral neurons. One of the factors has turned out to be identical to the leukemia inhibitory factor (LIF). It has a wide range of activity to control neurotransmitter/peptide expression not only in sympathetic neurons, but also in sensory neurons. Another factor induces only somatostatin expression and a third increases VIP peptide and/or acetylcholine levels.

These observations suggest the possibility that many distinct, diffusible factors can influence the development of the neurotransmitter/peptide phenotype in the peripheral nervous system. In addition, we recently found that the heart cell CM also has effects on neurotransmitter/peptide levels in cultured neurons prepared from the central nervous system (CNS). Subsequent biochemical analyses suggested that the responsible component resembles brain-derived neurotrophic factor (BDNF). BDNF increases and maintains the expression of putative inhibitory neuropeptides, neuropeptide Y, and somatostatin in cerebral cortical neurons.

Our finding demonstrates that as in the peripheral nervous system, neurotransmitter phenotypes in the CNS are plastic and can be regulated by many differentiation factors.

Our current efforts have been focused on the three projects: (1) identification of novel peptidergic differentiation factors, (2) characterization of their biological activity and physiological consequences, and (3) regulatory mechanism of their expression. These studies will elucidate the mechanism of how such a complex but highly ordered expression of neurotransmitters/peptides is controlled during development and how it affects neural plasticity such as learning and memory processes.

Characterization of VIP/Acetylcholine-inducing Activity

H. Nawa

The cholinergic differentiation factor (CDF) in heart cell CM is known to be identical to LIF. We have demonstrated that recombinant CDF/LIF alters dramatically neurotransmitter production as well as the levels of several neuropeptides (substance P, somatostatin, and VIP) in cultured rat sympathetic neurons. These changes are reflected by alteration in mRNA levels for these protein and peptides. Growth with 1 nM recombinant CDF/LIF induces mRNA for the adult form of choline acetyltransferase (ChAT) mRNA (4 kb) as well as for those peptides. It is known that cultured sympathetic neurons contain significant basal ChAT enzyme activity. RNA blotting experiments have shown that the original sympathetic ganglia do not contain a detectable amount of ChAT or VIP mRNA, whereas cultured sympathetic neurons grown with rat serum initiate expression of VIP mRNA and the embryonic form of ChAT mRNA (2.7 kb). After gel-filtration column chromatography, this activity in serum was recovered in fractions containing 60–100 kD. This suggests that the responsible factor is different from the known factors (LIF and ciliary neurotrophic factor [CNTF]), although further biochemical characteristics of the factor are unknown. Similar phenotypical alteration is observed in vivo: Sympathetic neurons innervating rat footpads change their neurotransmitter phenotype in response

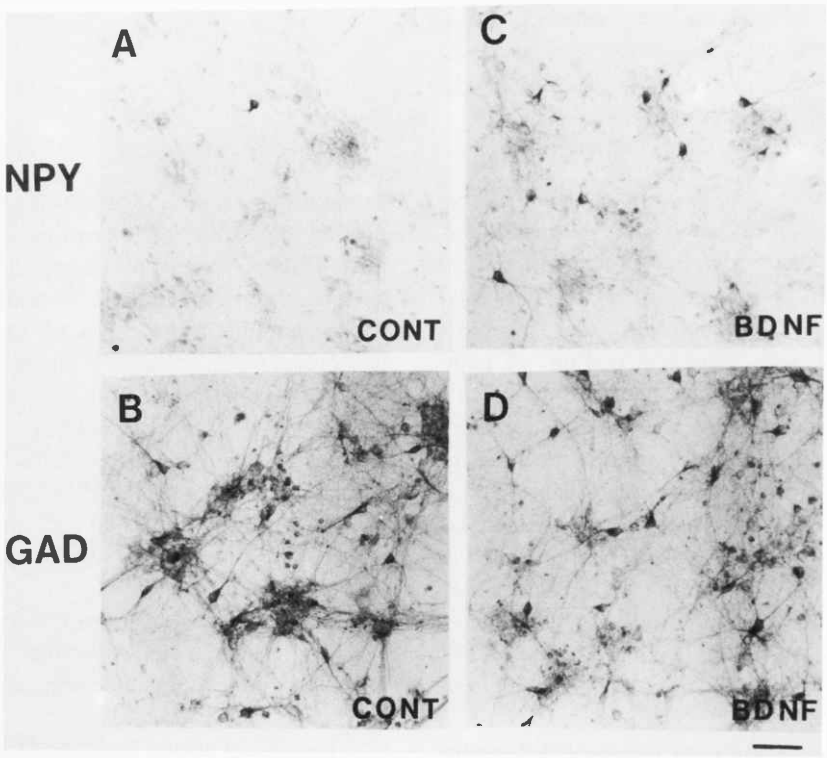

FIGURE 1 Immunocytochemical analyses of the BDNF effects on NPY- or GAD-positive neurons. Cortical neurons were grown for 5 days with (*C,D*) or without (*A,B*) BDNF. After treatment with 1 μM colchicine for 8 hr, neurons were fixed with 4% paraformaldehyde in 0.15 M phosphate buffer (pH 7.3) and immunostained with rabbit anti-NPY antiserum (1:600) (*A,C*) or sheep anti-GAD antiserum (1:1000) (*B,D*). The immunoreactivities were visualized with the biotinylated anti-rabbit IgG or biotinylated anti-sheep IgG (1:100), followed by the avidin-biotin-peroxidase complex (Vectastain ABC kit) (1:100). The frequency of GAD-positive neurons was 19.4 ± 2.6% in control cultures and 21.6 ± 2.2% in BDNF-containing cultures (n = 3). Bar, 100 μm.

to the signals from their targets. These neurons initially contain catecholamine and neuropeptide Y, but during postnatal life, they begin to express ChAT and VIP. Again, neither LIF nor CNTF seems to be responsible for this in vivo phenomenon. Therefore, these observations suggest that a novel factor induces VIP and/or ChAT in sympathetic neurons.

Plastic Regulation of Neuropeptide Y and Somatostatin Expression by BDNF

K. Mizuno, Y. Bessho, H. Nawa

The heart cell CM contains a large variety of neuronal trophic factors including LIF, FGF (fibroblast growth factor), IL-1 (interleukin-1), NGF (nerve growth factor), BDNF, and NT-3 (neurotrophin-3). Using the heart cell CM as a source of trophic factors, we are studying its effect on the neurotransmitter/peptide phenotype in cultured cerebral cortical neurons. These neurons were prepared from the neocortices of embryonic day-18 rats and received 25% heart cell CM during culture. Among the neuropeptides we examined, levels of the putative inhibitory neuropeptides, neuropeptide Y (NPY), and somatostatin were remarkably elevated by addition of heart cell CM. Further biochemical characterization of the responsible factor has shown that the activity is an extremely basic molecule whose size is less than 30 kD, suggesting that it may be one of the neurotrophins (including NGF, NT-3, and BDNF). We examined the peptidergic effects of these neurotrophins. BDNF increases the contents of NPY and somatostatin as well as their mRNAs without changing neuronal densities, but the other neurotrophins exhibited no effect. The level of NPY mRNA reached a maximum after 5 days addition of BDNF, but removal of BDNF on day 5 rapidly returned the level back to a control level. The delayed addition of BDNF until day 5 still increased the level of NPY mRNA in the same fashion as above. The fact that the delay did not alter the induction provides further evidence that this peptidergic differentiation activity of BDNF is independent of its trophic activity in promoting the neuronal survival reported previously. These neuropeptides are known to be colocalized with GABA or its synthetic enzyme GAD (glutamic acid decarboxylase) in the cerebrum. Immunocytochemical analyses have shown that treatment of the neurons with BDNF increased the frequency of NPY-positive neurons from 0.3% to 7.5% without changing that of GAD-positive neurons (Fig. 1). Moreover, almost all of the NPY-positive neurons induced by BDNF contain GAD immunoreactivity. These data suggest that BDNF regulates the neuropeptide phenotype of the GABAergic interneurons in a plastic manner. It is known that excitatory neurotransmission mediated by glutamic acid enhances and maintains BDNF expression. This observation, in conjunction with our results, suggests that excitatory neurotransmission might control the inhibitory neuropeptide levels in GABAergic neurons through BDNF.

Regulation of BDNF Expression in Neurons

Y. Bessho, H. Nawa

In the nervous systems, neuronal activity contributes to a variety of developmental neural processes such as synaptic elimination and stabilization, phenotypic differentiation, etc. We believe that some of these processes must be mediated by extracellular factors. We have been studying how neurotransmission regulates the production of neuronal differentiation factors in the nervous systems. We prepared primary cultures of cerebellar granule neurons from newborn rats and examined the influences of glutamatergic neurotransmission on BDNF expression. Four-hour exposure of the neurons to the glutamate receptor agonists, quisqualate, kainate, AMPA, and NMDA increased levels of BDNF mRNA, although some of the agonists produced neuronal degeneration. However, glutamate in combination with antagonists of the ionotropic glutamate receptors, CNQX, AP-5, and/or MK-801, still increased levels of BDNF mRNA. In contrast, the addition of glutamate to the cultures produced severe neuronal death and failed to increase the mRNA level. The onset of the increase in BDNF mRNA by kainate and NMDA significantly lagged behind that by quisqualate. These results indicate that the nonionotropic glutamate receptor is involved in the induction of BDNF mRNA. Quisqualate is known to be a potent agonist of both the ionotropic AMPA/kainate receptor and the metabotropic glutamate receptor. The specific antagonists of

the AMPA/kainate receptor, CNQX and DNQX, both failed to block the increase of BDNF mRNA by quisqualate. Moreover, the desensitization of the metabotropic glutamate receptor by phorbol ester abolished the increase of BDNF mRNA by quisqualate. These results suggest that stimulation of the metabotropic glutamate receptor is the most predominant component to increase BDNF mRNA in cerebellar granule cell culture. In contrast, NMDA produces the most drastic effect to increase levels of BDNF mRNA in cultured cortical neurons, and kainate induces BDNF mRNA most efficiently in cultured hippocampal neurons. All of these observations suggest that depending on neuronal cell type, the species of neurotransmitter or its receptor that control BDNF expression are different.

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GENE TARGETING AND THE BIOLOGY OF LEARNING AND MEMORY

A.J. Silva R. Burchuladze B. Frenguelli
C.M. Chen K.P. Giese

The key difficulty in the study of learning and memory is the *integration* of psychological, anatomical, and physiological information into a theory unrestrained by disciplinary boundaries. For example, physiologists are faced with the Herculean task of testing the relevance of candidate memory mechanisms in the behaving animal, and psychologists struggle to find physiological processes among the dwarfing complexity of the behaving brain! These methodological gaps result in theories of memory mostly defined within disciplinary boundaries.

Recent developments in mouse embryonic techniques allow the virtual molding of the mouse genome, and we propose that they will be key to the integration of neurobiological information with behavioral observations. Our laboratory has initiated an interdisciplinary study of mice mutant for genes that code for key components of hippocampal neural physiology. For example, with gene targeting, we have recently deleted the α isoform of the calcium/calmodulin kinase II (CaMkII) (Fig. 1). Electrophysiological studies of these mice revealed an impairment in the ability of the mice to establish a long-term potentiation of synaptic activity (LTP), a mechanism believed to be underlying learning and memory. Remarkably, other aspects of synaptic transmission seemed to be unaltered by the absence of this kinase. Behavioral studies of the mutant mice

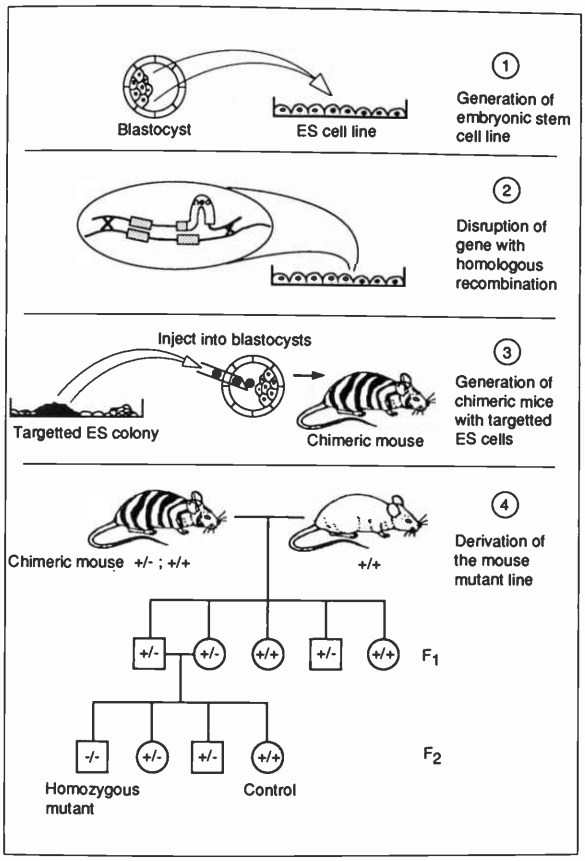

FIGURE 1 The gene-targeting procedure.

also showed a surprising specificity of the affected behaviors. For example, we found that spatial learning was severely disrupted, whereas other learning processes were not. The results demonstrated that this genetic approach can yield relatively specific function lesions in the brain, and they were important in directing our work: First, these results showed that the approach works, and we have applied it to other candidate genes. Second, they also demonstrated that the disruption of αCaMkII interfered with hippocampal-dependent learning, providing us with the opportunity to determine how this happens. Third, these results motivated us to look within other analytical levels, and we have recently found that neural circuits in the mutants react very differently to low-level electrical stimulation delivered according to an experimented paradigm referred to as kindling. Our kindling experiments (in collaboration with Jim McNamara) uncovered a role for CaMkII in the regulation of the excitability of neural circuits, and they raised the possibility that this role might underlie the deficits in LTP and learning found in the αCaMkII mutant mice.

In the last 4 months of 1992, our laboratory has ordered equipment and swelled ranks, and we actually did get some work done.

Rusudan Burchuladze has set up the Morris water maze in our behavioral room. The christening of the pool was done with mice mutant for the neurofibromatosis type I (NF1) gene (Fig. 2). In humans, the mutation of this gene is the second most common cause for learning disabilities. These cognitive impairments appear to be the most disabilitating consequence of this common genetic disorder. Surprisingly, our studies of both spatial and visual learning and memory detected no abnormalities in mice heterozygous for the NF1 gene, with the possible exception that the rate of learning might be slower in the NF1 mutant mice. We must acknowledge the great support personnel at Cold Spring Harbor Laboratory. Their thoughtful, and at times inspired, suggestions have contributed immensely to the numerous experimentally relevant and time-saving improvements introduced in our water maze.

Chua Min Chen has developed a targeting vector that we will use to introduce a substitution of Thr-286 (CGG) for alanine (CAG) in αCaMkII. The vector design is based on the "hit-and-run" procedure developed by Alan Bradley (Fig. 3). In our previous mutants, the complete loss of αCaMkII not only eliminated the kinase activity of the enzyme, but also eliminated all other possible functions that such an abundant component of postsynaptic densities might have. For example, it is possible that the αCaMkII is not only a kinase, but also an essential structural component of spines and even synaptic vesicles. Thus, the learning impairment could be unrelated to LTP and be solely due to the potential disruption of postsynaptic densities. In Thr-286 mutant mice, the

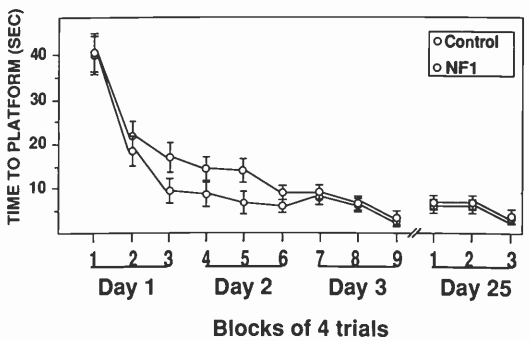

FIGURE 2 NF1 mutants: The hidden platform task.

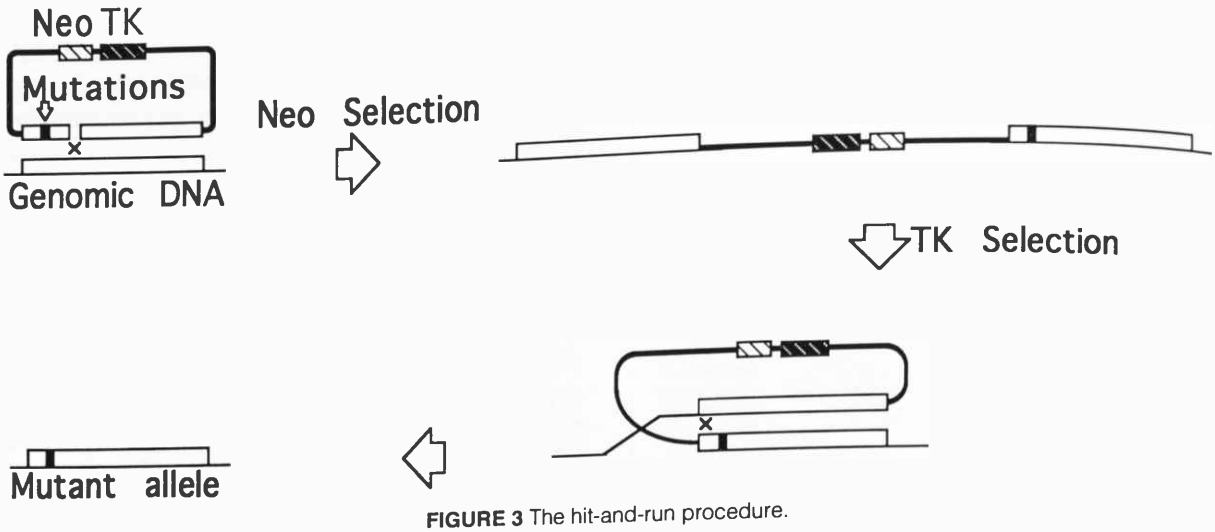

FIGURE 3 The hit-and-run procedure.

structure of the enzyme should be basically undisturbed, and all of the Ca/CaM-independent kinase activity should be eliminated. We are anxious to determine whether this mutation disturbs LTP and learning to the same extent as the null mutation.

Karl Peter Giese recently joined our laboratory and will continue the derivation of mice mutant for the Thr-286 of αCaMkII. Additionally, he will derive other mutations in components of hippocampal physiology. Bruno Frenguelli will continue the electrophysiological characterization of our mutant mice. Next year, we hope to have an exciting account of our genetic journey through the behavior and physiology of learning and memory.

Finally, I must confess that Cold Spring Harbor Laboratory is much more than what I expected, and I did expect a lot. Overall, these last 4 months have been wonderful, and we have been fortunate to put together a laboratory with an enthusiastic group that will be able to generate a "learning" mutant and immediately characterize its physiology and behavior. And thus we forge ahead with the conviction and naive arrogance that only new but promising approaches allow.

SIGNAL TRANSDUCTION IN NEURONS

G. Enikolopov N. Peunova

We study how signal transduction and gene expression in neurons are involved in synaptic plasticity. Signals that ultimately produce changes in synaptic plasticity can also elicit modifications of gene activity patterns in neurons. Induced changes in gene activity may underlie both antero- and retrograde transfer of information and may in turn be required for long-term changes in neuronal function. We are particularly interested in molecular mechanisms that permit a neuron to integrate coincident signals and to relay them to other cells. This year, our efforts concentrated on the action of nitric oxide, a new type of cellular messenger. We found that nitric oxide can act as an amplifier of calcium signals in neuronal cells.

Amplification of Calcium-induced Gene Transcription by Nitric Oxide in Neuronal Cells

N. Peunova, G. Enikolopov

Nitric oxide (NO) is a short-lived, highly reactive gas with free radical properties, which has been identified as a mediator in vasodilation, an active agent in macrophage cytotoxicity and in neurotoxicity, and a neurotransmitter in the central and peripheral nervous systems. Production of NO by neurons is critical for facilitated synaptic transmission in models of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), suggesting a role for NO as a "retrograde messenger" that could complete a hypothetical feedback loop by strengthening the connection between postsynaptic and presynaptic cells. We have found that although NO itself has no evident effect upon transcription, it can synergistically enhance calcium-mediated signals in neuronal cells.

A potential problem with the concept of an easily diffusible messenger is the possibility of nonspecific activation of many different terminals in the vicinity of an active synapse, which would contradict the known specificity of most examples of synaptic plasticity. One solution to this paradox is restriction of the effect of NO to cells that are somehow marked by another event. For example, in the nervous system, the response to NO might be limited to recently active neurons or synapses, thereby coinciding with transient elevations of calcium levels. In an attempt to model a situation in which a neuron receives an NO signal simultaneously with a signal of a different modality, we exposed neuronal PC12 cells to combinations of NO with different inducers of transcription and monitored changes in immediate-early gene transcription. NO was unable to induce transcription on its own; however, when applied to cells in combination with certain treatments known to induce immediate-early gene expression, induction of c-*fos* expression was greatly enhanced. Importantly, NO only amplified the action of those agents that act through calcium ions. The elevated induction of c-*fos* mRNA was productive, leading to an increase in binding activity of the AP-1 transcription factor comprising Fos and Jun family proteins. Our experiments with pulse application of one inducer followed by addition of the second component indicate that the two stimuli, NO and calcium, must be present together within a short time window in order to exert their synergistic action.

Transfection experiments with c-*fos*-CAT constructs indicate that a short (400 bp) promoter region of the c-*fos* gene is sufficient to confer NO/Ca^{++} inducibility. Using a series of promoter mutation constructs and chimeric GAL4-CREB activators (gifts from Dr. M. Gilman), we have demonstrated that CRE sequences and the transcriptional activator CREB are targets for at least one (and, probably, the major) of the NO/Ca^{++}-induced pathways in the nucleus.

Ca^{++} signaling in neurons is mediated by several serine/threonine-specific protein kinases. To determine which enzymes are involved in signaling by NO/Ca^{++}, we have used specific recombinant inhibitors of individual protein kinases. These inhibitors were constructed based on the autoinhibitory pseudosubstrate domains of several protein kinases and were shown to be potent and selective toward their cognate enzymes. The results of such analysis suggest that, consistent with the data on promoter mapping, only the cAMP-dependent protein kinase (PKA) inhibitor and, to a small degree, Ca^{++}/calmodulin-dependent protein kinase (CaMK) inhibitor blocked the NO/Ca^{++} response. These results implicate the PKA-CREB-CRE system as a major component of the signaling pathway for the transcriptional synergy of NO and Ca^{++}.

The phenomenon of NO-mediated potentiation of the Ca^{++} response may have implications beyond transcriptional regulation. For example, NO could potentiate the PKA phosphorylation of cytoplasmic proteins with direct roles in synaptic funtion. The effect of NO on signaling might be particularly important at very low levels of calcium action, at which this inducer acting alone would have negligible effect; these very weak signals, which would go unnoticed by the cell, might be amplified by NO, resulting in pronounced physiological changes for the cell. Since NO diffuses freely and readily leaves and enters cells without need for a secretory system or a surface receptor, nearby synapses that receive very weak impulses simultaneously with exposure to NO might establish facilitated synaptic transmission, a phenomena that is known for LTP and which may form the basis for associative memory. NO and Ca^{++} must act within a very narrow time window for this

enhancement to occur, suggesting that in the nervous system, this synergistic effect might be restricted to the recently active synapses. Amplification of Ca^{++} action by NO, acting on signaling machinery either directly or through reprogramming of gene activity, might be directly involved in NO-mediated steps in LTP and similar activity-dependent neuromodulation phenomena in the brain.

In Press, Submitted, and In Preparation

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NEUROBIOLOGY AND NEUROGENETICS OF SYNAPTIC PLASTICITY

Y. Zhong J. Shanley
N.J.D. Wright
Z. Xie

The primary focus of our laboratory is on molecular mechanisms and physiological functions of synaptic plasticity. Synaptic plasticity is referred to as those processes that modify the strength of synaptic transmission in a neural activity-dependent manner. Various processes including facilitation, potentiation, and depression are exhibited by synaptic plasticity. These plastic processes have been suggested as a cellular basis of learning and memory. Thus, it would be expected that a mutation that causes learning or memory deficiency may also alter synaptic plasticity. Therefore, we are taking a major approach to examine synaptic transmission in *Drosophila* learning and memory mutants. The defective synaptic plasticity identified in these mutants will help not only to frame whether and how synaptic facilitation, potentiation, and depression are involved in learning and memory processes, but also to determine biochemical components underlying the disrupted synaptic plasticity.

Synaptic Plasticity in Learning vs. Memory Mutants

Y. Zhong

About 15 to 20 different lines of learning and memory mutants have now been isolated and more are expected. Some of these mutants are classified as learning mutants, and they show profound defects in their initial learning scores, although the decay rate of this learning appears to be similar to that in normal flies. In contrast, memory mutants exhibit a significantly different decay rate of learning scores after training.

I have examined short-term potentiation at neuromuscular junctions of some of these mutants by the two-electrode voltage-clamp method. Preliminary results indicated that short-term potentiation was abolished in memory mutants, including *rutabaga* (Fig. 1), *turnip*, and *radish*, whereas potentiation was observable but altered in learning mutants such as *dunce*, where it was much shorter (Fig. 1), and in *latheo* mutants, where the size was reduced. In addition to examination of this short-term potentiation, I will also study facilitation, depression, and a long-term potentiation in all available mutants in an attempt to determine the differences, if any, in alteration of synaptic plasticity induced by learning mutations vs. memory mutations. Although obtained from the neuromuscular junctions, these data reflect the functions of genes important for learning ability in synaptic transmission, in contrast to those for retention of learned behavior. In addition, there is also the possibility of dissecting learning and memory neurobiologically.

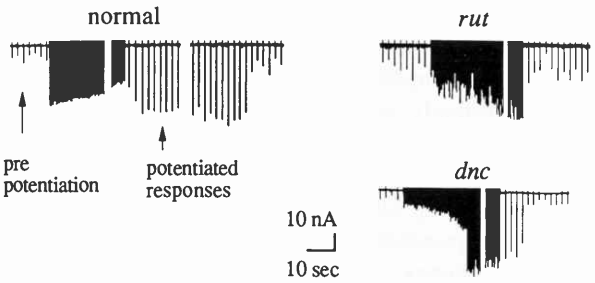

FIGURE 1 Short-term potentiation in different genotypes. Synaptic currents were recorded at the larval neuromuscular junctions. Test frequence is 0.5 Hz, tetanic stimulation is 5 Hz. Normal larvae exhibit potentiation lasting from 2 min to 15 min, whereas *dnc* shows less than 10 sec and *rut* eliminates this potentiation.

Function of the *latheo* Peptide

Y. Zhong [in collaboration with T. Tully, Cold Spring Harbor Laboratory]

The learning mutant *latheo* has been isolated by Tim Tully's laboratory. In addition to its phenotype of a reduced short-term potentiation as mentioned above, the mutant also exhibited hyperexcitability as indicated in Figure 2. First, its basal level of postsynaptic currents was increased (comparing the first evoked response of *latheo* to that of normal larvae in Fig. 2), indicating enhanced synaptic transmission. Second, a single stimulus was able to induce multiple discharges in *latheo* instead of the single discharge seen in normal larvae (Fig. 2). This hyperexcitability could be mimicked by application of K^+ channel blockers to normal larvae, suggesting that the *latheo* locus may encode a K^+ channel subunit or a modulator of K^+ channels. It is interesting that the modulation of K^+ channels provides an essential molecular mechanism for mediating sensitization and associative learning in *Aplysia* and *Hermissenda*. It may also be important for induction of long-term potentiation in the hippocampus.

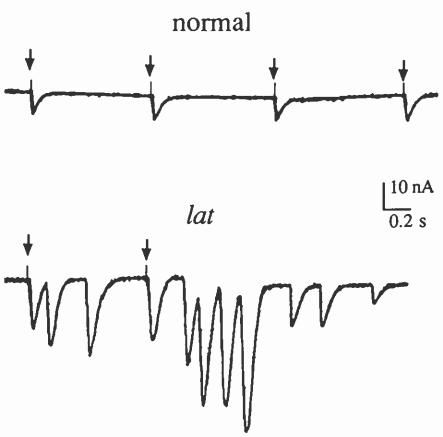

FIGURE 2 Effect of *lat* mutation on synaptic transmission. Excitatory junctional currents were recorded from the body-wall neuromuscular junctions of third instar larvae at 0.2 mM Ca concentration. Arrows point to artifacts of electric stimuli. The *lat* mutation induces multiple discharges in response to single nerve stimulus.

Membrane Currents in Mushroom Body Neurons

N.J.D. Wright, Z. Xie, Y. Zhong

The mushroom body is an area in the brain thought to be important for learning and memory in insects including *Drosophila*. Work in Ron Davis' laboratory (see this section) has revealed that several genes important for fly learning and memory such as *dunce* and *rutabaga* are predominately expressed in this region. They have also isolated several enhancer-detector lines in which *lacZ* is expressed exclusively in the mushroom body. These lines allow us to identify the mushroom body neurons in culture by fluorescent staining of the living cells. Membrane currents in these stained cells can then be analyzed by the patch-clamp method. Surprisingly, currents recorded from these neurons appeared to be very similar (Fig. 3), in sharp contrast to the diverse types of currents recorded from nonstained neurons. Such uniformity provides a basis for the genetic analysis of these membrane currents in neurons that may participate in learning processes of mutants. In light of the *latheo* phenotype as discussed above, and of predominant expression of a cAMP-specific phosphodiesterase encoded by the *dunce*, an adenylyl cyclase by the *rutabaga* locus, a protein kinase A, and a K^+ channel subunit by the *Shaker* locus in the mushroom body, we would like to postulate that modulation of K^+ currents by the cAMP cascade is essential for the physiological functions of the mushroom body neurons. We are examining the effects of the *Shaker* and *latheo* mutations on K^+ currents in identified neurons and probing their relation to modulation of the cAMP cascade.

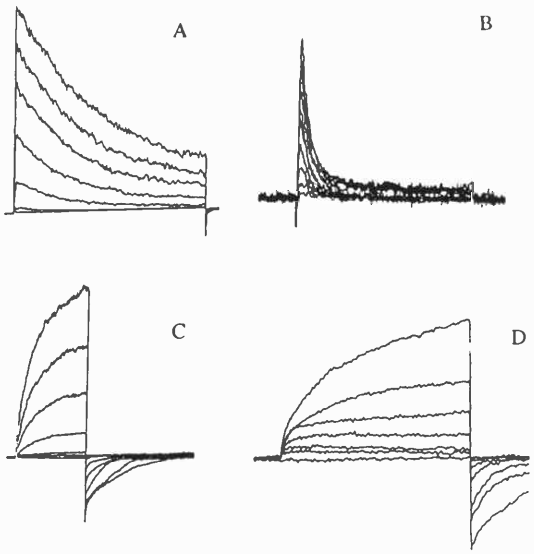

FIGURE 3 Whole-cell voltage-clamp recordings of membrane currents from the cultured larval CNS neurons. (*A–C*) Currents from nonstained neurons; (*D*) a representative current recorded from the stained mushroom-body neuron.

Role of Cell Adhesion Molecules in Synaptic Plasticity

Y. Zhong, J. Shanley

The number of cell adhesion molecules is regulated during induction of long-term facilitation in *Aplysia*, and application of antibody against a cell adhesion molecule blocks the later phase of long-term potentiation in the hippocampus. Therefore, it is suggested that regulation or modulation of cell adhesion molecules may lead to the formation of new synapses, which in turn may enhance synaptic transmission. However, direct evidence is not yet available to support this hypothesis. The existence of *Drosophila* mutants has allowed us to approach this problem more directly. Immunohistochemical staining of the neuromuscular preparation of the mutant *fasciculin I* (*fas I*), which encodes a cell adhesion molecule, revealed that the numbers of nerve terminal branches and varicosities (corresponding to synapses) were increased in this null mutant as compared to that in normal nerve terminals (Fig. 4). Conversely, the numbers of branches and varicosities were reduced in flies with an extra copy of the *fas I* gene (*Dp* [*fas I*]). These results indicate that indeed the regulation in the number of cell adhesion molecules may lead to changes in the number of synapses formed. However, further electrophysiological analysis of these have provided a very intriguing result. The amplitude of postsynaptic currents was not accordingly enhanced in *fas I* mutants, which showed more synapses as compared to normal flies. Instead, synaptic currents were enhanced significantly in *Dp* (*fas I*). Further examination is under way to probe the possible mechanisms and significance of such phenomena.

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In Press, Submitted, and In Preparation

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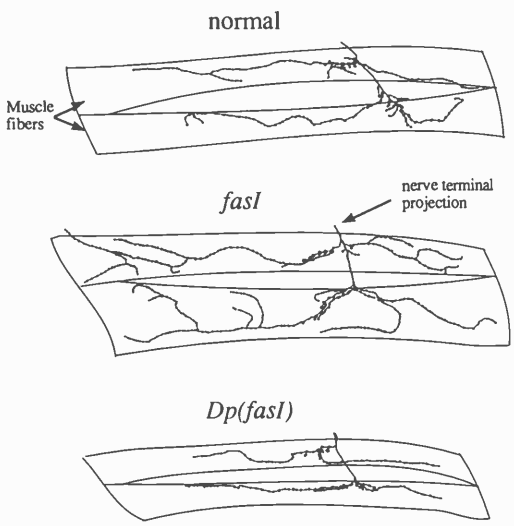

FIGURE 4 Effects of mutations of the *fas I* gene on the nerve terminal arborization. The *fas I* mutant is a null mutation that enhances the nerve terminal arborization. The *Dp* (*fas I*) fly has an extra copy of the *fas I* gene that reduces the numbers of branches and varicosities.

CSH LABORATORY JUNIOR FELLOWS

In 1986, Cold Spring Harbor Laboratory began a Junior Fellow program to encourage independent research by outstanding young scientists who, during their graduate studies, displayed exceptional promise of becoming leading scientists of the future. The purpose of this program is to provide an opportunity for each Fellow to work independently at the Laboratory for a period of up to 3 years on projects of their choice. The Fellow is provided with a salary, research support, and technical assistance so that they can accomplish their goals free from other distractions. The interaction among research groups at the Laboratory and the program of courses and meetings on diverse topics in molecular biology contribute to a research environment that is ideal for innovative science by these Fellows.

Three previous Cold Spring Harbor Fellows, Dr. Adrian Krainer (1987), Dr. Carol Greider (1988), and Dr. Eric Richards (1989), are currently members of the scientific staff at the Laboratory. Dr. David Barford is our most recent Fellow, having joined us in 1991 from Professor Louise Johnson's laboratory at Oxford. Dr. Barford is an X-ray crystallographer who has previously worked on glycogen phosphorylase, but whose present interests lie in the area of protein-mediated signal transduction. Dr. Barford's current focus is on the serine/threonine-specific protein phosphatases. Immediately before coming to Cold Spring Harbor, Dr. Barford spent some time in Dr. P. Cohen's laboratory in Dundee working on protein phosphatase 2A (PP2A) from rabbit muscle. Dr. Barford hopes to crystallize and solve the structure of this phosphatase.

D. Barford

Structural Studies on Protein Phosphatases

D. Barford

The aim of my research is to determine the three-dimensional structure of protein phosphatases using X-ray crystallography. This information will be useful in describing the overall conformation of the protein and the mechanism of catalysis and substrate specificity. This year, progress has been made in cloning, expressing, purifying, and crystallizing three protein phosphatases: human protein phosphatase 1 (PP1), human T-cell protein tyrosine phosphatase (PTPase), and a bacteriophage λ phosphatase. PP1 is representative of the serine/threonine class of protein phosphatases, PTPase is a tyrosine-specific protein phosphatase, and the bacteriophage phosphatase is a dual specificity phosphatase. The latter phosphatase shares considerable sequence identity with the amino-terminal 115 amino acids of PP1 (35% sequence identity), hence the finding that it possesses activity against phosphotyrosine was unexpected.

CLONING, EXPRESSION, PURIFICATION, AND CRYSTALLIZATION OF PROTEIN PHOSPHATASES

Human Protein Phosphatase 1 (in collaboration with Dr. P.T.W. Cohen, Dundee, United Kingdom). The cDNA encoding human PP1γ was cloned into the *Escherichia coli* expression vector pTactac. *E. coli* cells grown at 30°C express appreciable quantities of active soluble PP1. The protein is purified to homo-

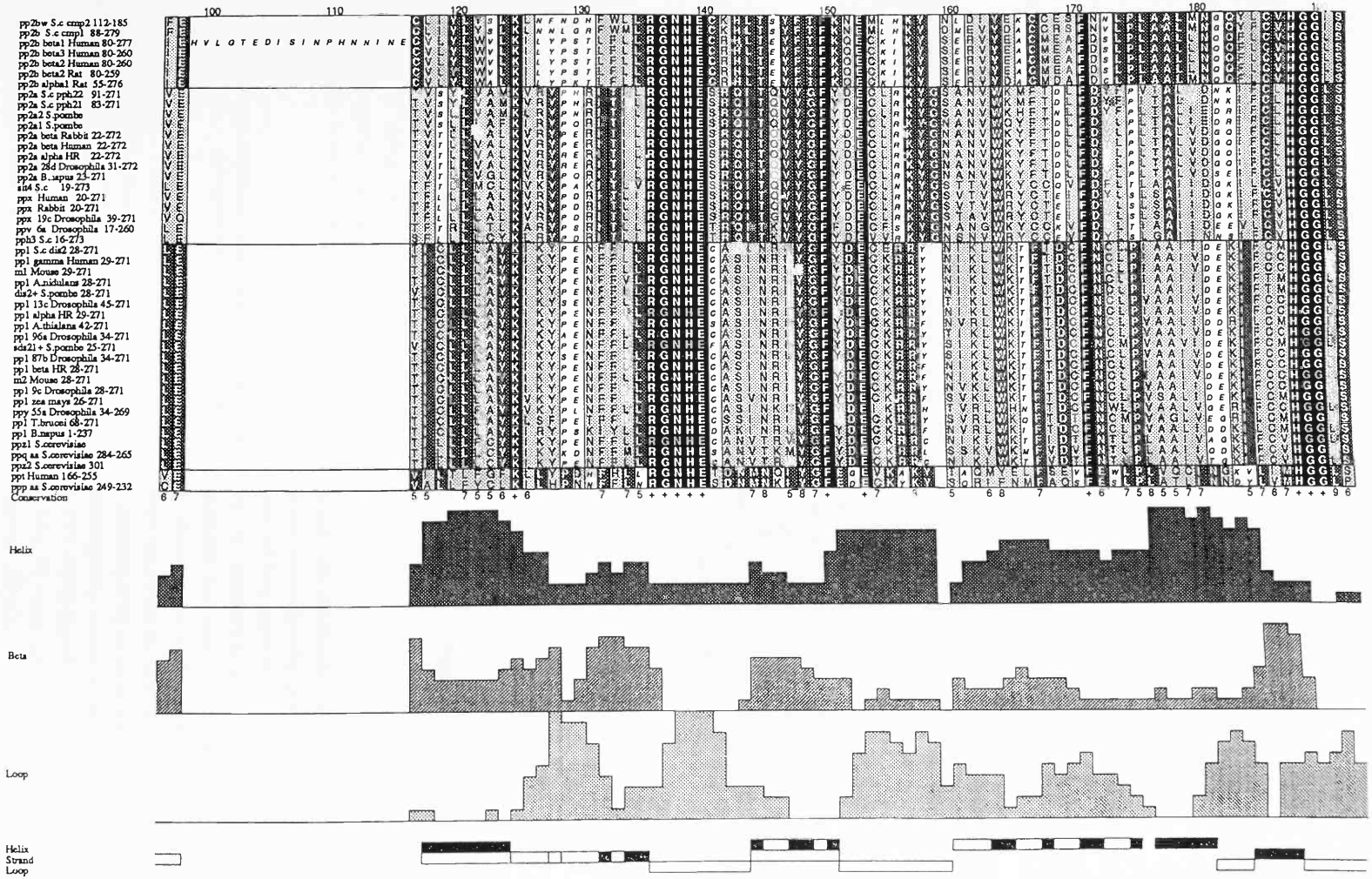

FIGURE 1 Portion of the multiple sequence alignment of 45 serine/threonine phosphatases. The family is divided into four classes: PP2B, PP2A, PP1, and PPP/PPT. The extent of conservation within the family and within subfamilies is indicated by shading. (*White letters on black*) Invariant residues throughout the family; (*white letters on dark gray*) invariant residues within a family; (*black letters on light gray*) physiochemically related residues within a subfamily; (*black letters on white*) positions where there is no sequence conservation. The combined secondary structure predictions for each sequence is shown below the sequence alignment, and the proposed secondary structure assignment is indicated.

geneity using heparin agarose, mono-Q FPLC, and gel-filtration chromatography. The purified protein has been subject to crystallization screening and conditions have been discovered where the protein will crystallize. The crystals diffract to 2.9 Å and are suitable for structure determination.

Human T-cell Protein Tyrosine Phosphatase (in collaboration with Dr. N. Tonks, Cold Spring Harbor Laboratory). The cDNA encoding the amino-terminal 321 amino acid residues of the human T-cell PTPase has been cloned into the *E. coli* T7 expression vector. As much as 10–20 mg of protein can be purified from 2 liters of bacterial culture using DEAE Sepharose, mono-S FPLC, and phenyl TSK hydrophobic interaction chromatography. The protein crystallizes to form needles of length 1 mm by 0.02 mm by 0.02 mm. Attempts are being made to improve the thickness of these crystals to allow crystallographic data to be collected.

Bacteriophage λ ORF221 Protein Phosphatase (in collaboration with Dr. J.E. Dixon, Michigan University). The cDNA encoding the bacteriophage ORF221 was cloned into the T7 expression vector. Large quantities of protein are produced that enable kinetic and structural analyses to be performed. This has demonstrated phosphoserine as well as phosphotyrosine activities. The protein has been crystallized and diffracts to better than 4 Å with crystal cell dimensions, a = 80.8 Å, b = 193.2 Å, c = 53.9 Å, β = 93.3°. Structural studies are in progress.

MULTIPLE SEQUENCE ALIGNMENT OF SERINE/THREONINE PHOSPHATASES

In addition to efforts to determine the three-dimensional structures of proteins by X-ray crystallography, we have taken advantage of the large number of cDNA sequences available for the serine/threonine phosphatase family. A multiple sequence alignment of a family of related proteins yields information about the position of invariant residues (important in catalysis, substrate recognition, regulation, and protein-folding determinants) and the positions of insertions and deletions (which can only be tolerated at surface loops and turns). By combining secondary structure predictions at each position for all aligned sequences, a more accurate consensus secondary structure prediction can be obtained. The pattern of conserved residues also provides clues to the secondary structure. For example, conserved hydrophobics at positions i, i+3, i+4, i+7 is suggestive of an α-helix with a hydrophobic surface buried in the protein core.

In collaboration with Dr. P.T.W. Cohen (Dundee University) and Dr. Geoff Barton (Oxford University), 45 serine/threonine phosphatase sequences have been gathered and multiply aligned. The consensus sequence produced from the alignment matches the amino-terminal 100 residues of an *E. coli* diadenosine tetraphosphatase, with conservation of invariant eukaryotic residues. The similarity among eukaryotic serine/threonine phosphatases, a bacteriophage phosphatase, and an *E. coli* diadenosine tetraphosphatase raises interesting questions about the evolutionary and structural relationships of these proteins. A representation of the results is shown in Figure 1.

The study has revealed the positions of invariant residues, likely to be important in catalysis and has allowed us to propose a secondary structure prediction. It will be of interest to compare the crystal structure of a serine/threonine protein phosphatase with this prediction.

PUBLICATIONS

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In Press, Submitted, and In Preparation

Barton, G.J., P.T.W. Cohen, and D. Barford. 1993. Conservation analysis and structure prediction of Ser/Thr phosphatases. Diadenosine tetraphosphatase from *E. coli* is homologous to protein phosphatases. (Submitted.)

Johnson, L.N. and D. Barford. 1993. The effects of phosphorylation on the structure and function of proteins. *Annu. Rev. Biophys. Biomol. Struct.* (in press).

Zhou, S., J.C. Clemens, D.J. Hakes, D. Barford, and J.E. Dixon. 1993. Expression, purification, crystallization, and biochemical charaterization of a recombinant protein phosphatase. (In preparation.)

Author Index

COLD SPRING HARBOR MEETINGS

ACADEMIC AFFAIRS

The academic program at Cold Spring Harbor Laboratory includes a wide-ranging series of postgraduate laboratory and lecture courses, workshops, large meetings, and a summer research program for undergraduates. The program extends from a spring session of courses early in April through a fall session of courses ending early in November. In 1992, 21 courses in molecular genetics and neurobiology and 14 meetings were held. The academic program continues to expand, and several new meetings and courses took place this year. Two new fall courses were held: Monoclonal Antibodies from Combinatorial Libraries (taught by Carlos Barbas and Dennis Burton) and Molecular—Cell Biology Techniques: Immunocytochemistry and In Situ Hybridization (taught by Paul Hough, Ken Jacobson, David Spector, and Barbara Trask). The neurobiology courses were moved from Jones Laboratory to the new Howard Hughes Medical Institute (HHMI) teaching laboratories on the top floor of the Beckman Neuroscience Center. Although many instructors were wary of leaving Jones Laboratory with its familiar waterfront location, they quickly came to appreciate the state-of-the-art facilities in the HHMI laboratories.

The courses are supported by a series of grants from public and private sources. For many years, the summer molecular genetics courses have been made possible by grants from the National Institutes of Health (NIH) and the National Science Foundation (NSF). Grants from the NIH support several of the newer spring and fall courses, and funds from a grant from the National Institute of Mental Health cover several neurobiology courses. 1992 represented the first full year of the Laboratory's renewed education grant from HHMI. This award has provided stable funding for the neurobiology program as well as support for new courses, allowing the Laboratory flexibility in initiating courses. The Laboratory also has an award from the Klingenstein Fund and the Grass Foundation for the support of the neurobiology program. In addition, the Laboratory courses receive invaluable aid from many companies (see Educational Activities Section) that donate supplies and provide large amounts of equipment on loan.

Conferences at the Laboratory have grown to 14 this year, including meetings that cover new topics and those that are held on a yearly or biannual basis. The annual Symposium continues to be a highlight of the year. This year's Symposium, The Cell Surface, was organized by Richard Hynes, Richard Axel, Corey Goodman, and Bruce Stillman. Several new meetings, e.g., The Cell Cycle and Gene Therapy, were received very enthusiastically and it was decided to hold them again in 1994. Although many of the meetings, including the conferences on Genome Mapping and Sequencing and RNA Tumor Viruses, were oversubscribed, the new and renovated facilities comprising Grace Auditorium, Blackford Hall, Dolan Hall, and the new cabins made all aspects of the meetings more pleasant and efficient. The success of the meetings is largely due to the efforts of all of the scientists who serve as organizers and to the enthusiastic participation of all of the visiting scientists.

In these times of concern about grants and funding, support for the meetings program from the Laboratory's Corporate Sponsor Program and from NIH, NSF, and the Department of Energy was extremely important. In fact, this year, the Gene Therapy meeting was funded by a most generous grant from the Wellcome Trust and the Translational Control meeting was supported by funds from ICN Pharmaceuticals, Inc.

While graduate students, postdoctoral fellows, and faculty participate in the courses and meetings, the Undergraduate Research Program (URP) provides an opportunity for college undergraduates to spend 10 weeks at the Laboratory during the summer. The program, headed by Winship Herr, allows students to do research in the laboratories of staff scientists.

That the meetings and courses proceed with such efficiency and good spirit is due, in large measure, to the skill and hard work of the staff of the Meetings Office: Barbara Ward, Micki McBride, Diane Tighe, Eileen Paetz, Marge Stellabotte, Andrea Stephenson, and Nancy Weeks. The audiovisual staff headed by Herb Parsons manages with great efficiency and good humor the thousands of slides and projections provided by the scientists at our meetings. The course instructors have come to depend on the skills of Cliff Sutkevich and his staff in setting up and maintaining all of the necessary equipment and supplies. It is a necessity to obtain and manage the many grants that support the academic program, and Mary Horton of the Grants Office has ably coordinated these efforts. The success of the academic program is the result, to a great extent, of the work and assistance of Laboratory staff from all areas—from scientists who give lectures (and sometimes teach) in the courses, to those in the Purchasing Office (Sande Chmelev, Pat Hinton, Mary Ellen Fredericksen, and Barbara Zane), to staff in the Buildings and Grounds Department and Blackford Hall.

Terri Grodzicker
Assistant Director for Academic Affairs

57th COLD SPRING HARBOR SYMPOSIUM ON QUANTITATIVE BIOLOGY

The Cell Surface

May 27–June 3, 1992

ARRANGED BY

Richard Axel, HHMI, Columbia University
Corey Goodman, University of California, Berkeley
Richard Hynes, Massachusetts Institute of Technology
Bruce Stillman, Cold Spring Harbor Laboratory

321 participants

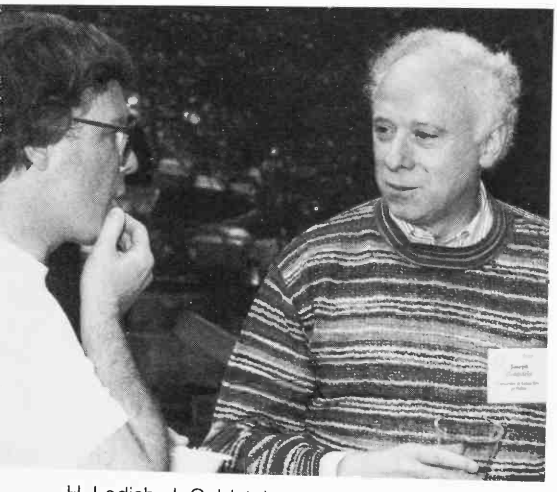
The biochemical processes that occur at the cell surface have profound effects on the well-being of single cells and whole organisms. Cell-surface phenomena are principal areas of study in all disciplines of modern biological research, including physiology, development and differentiation, immunology, cancer, neurobiology, and cell growth and death. Moreover, the molecules that lie on or immediately subjacent to the cell surface comprise prime targets for alleviation or treatment of human diseases. Because of the importance of this research, it was decided to focus the 57th Cold Spring Harbor Symposium on the topic of The Cell Surface. It was a considerable challenge, however, to attempt to bring together a diverse group of scientists who study biology at the cell surface. But there was also comfort in the fact that common molecular themes appear in all species, from bacteria to humans.

M. Brown

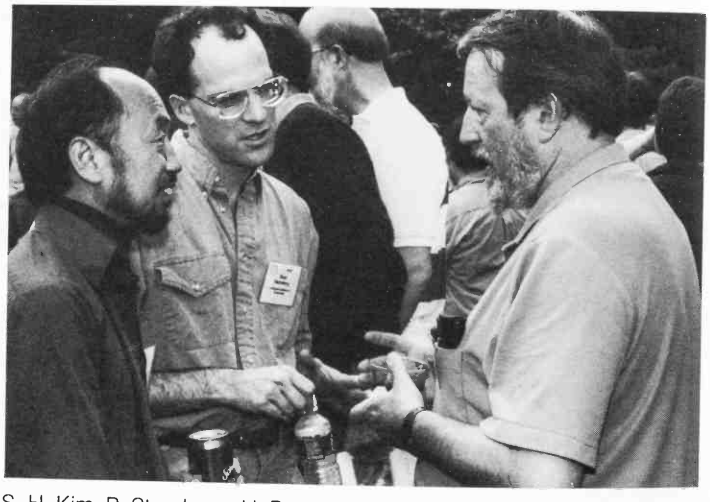
G. Stiller, B. Stillman, D. Wiley, D. Luke III, H. Horwitz

The very first Symposium organized by Reginald Harris in 1933 was entitled Surface Phenomena and in the intervening years, many other Symposia have dealt with many aspects of this broad topic. In 1992, given the breadth of the program, it was not possible to invite all the major scientists working in this field and so difficult decisions were necessary. Following an introduction by Jim Watson, the first-night speakers, Christiane Nüsslein-Volhard, Dan Koshland, Jr., Tony Hunter, and Gerald Edelman provided a fascinating overview and a taste of what was to come. During the 6 days, 321 participants witnessed a total of 82 oral presentations and 89 posters and the high standard set on the first night was maintained throughout the meeting. An insightful and wonderful summary was presented by Mel Simon.

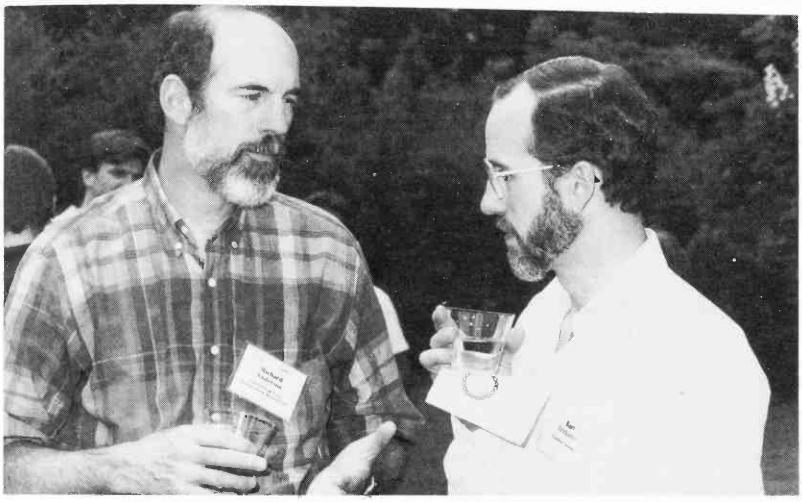
Essential funds to hold such a meeting were provided by the National Science Foundation, the U.S. Department of Energy, Office of Health and Environmental Research, and the following divisions of the National Institutes of Health: the National Cancer Institute, the National Institute of Neurological Dis-



H. Lodish, J. Goldstein



S.-H. Kim, P. Sternberg, H. Bourne



R. Anderson, K. Drickamer

M. Simon

orders and Stroke, and the National Institute on Aging. Additional funding came from our Corporate Sponsors who provide increasingly essential funding: Akzo Pharma International B.V.; Alafi Capital Company; American Cyanamid Company; Amgen Inc.; Applied Biosystems, Inc.; BASF Bioresearch Corporation; Becton Dickinson and Company; Boehringer Mannheim Corporation; Ciba-Geigy Corporation/Ciba-Geigy Limited; Diagnostic Products Corporation; The Du Pont Merck Pharmaceutical Company; Eastman Kodak Company; Genentech, Inc.; Glaxo; Hoffmann-La Roche Inc.; Johnson & Johnson; Kyowa Hakko Kogyo Co., Ltd.; Life Technologies, Inc.; Eli Lilly and Company; MetPath Inc.; Millipore Corporation; Monsanto Company; New England BioLabs, Inc.; Oncogene Science, Inc.; Pall Corporation; Perkin-Elmer Cetus Instruments; Pfizer Inc.; Recordati; Sandoz Research Institute; Schering-Plough Corporation; SmithKline Beecham Pharmaceuticals; Sumitomo Pharmaceuticals Co., Ltd.; Toyobo Co., Ltd.; The Upjohn Company; The Wellcome Research Laboratories,; Burroughs Wellcome Co.; and Wyeth-Ayerst Research.

M. Mathews, R. Hynes

C. Goodman

J. Brugge

PROGRAM

Welcoming Remarks: J.D. Watson

Introduction: The Cell Surface
Chairperson: R.O. Hynes, *HHMI, Massachusetts Institute of Technology*

Receptors
Chairperson: H.R. Bourne, *University of California, San Francisco*

Regulation at the Cell Surface
Chairperson: A.G. Gilman, *University of Texas Southwestern Medical Center, Dallas*

Development
Chairperson: C.S. Goodman, *HHMI, University of California, Berkeley*

Immunology
Chairperson: S. Tonegawa, *HHMI, Massachusetts Institute of Technology*

Neurobiology
Chairperson: L. M. Reichardt, *HHMI, University of California, San Francisco*

Morphogenesis and Pattern Formation
Chairperson: J. Kimble, *University of Wisconsin, Madison*

Cell Adhesion
Chairperson: E. Ruoslahti, *La Jolla Cancer Research Foundation*

Receptors in the Vascular System
Chairperson: J.S. Brugge, *HHMI, University of Pennsylvania, Philadelphia*

Dorcas Cummings Lecture
Speaker: M. Brown, *University of Texas Southwestern Medical Center, Dallas*

Transduction of Signals
Chairperson: J.L. Goldstein, *University of Texas Southwestern Medical Center, Dallas*

Cell Migration and Axon Guidance
Chairperson: H.R. Horvitz, *HHMI, Massachusetts Institute of Technology*

Membrane Traffic and Organization
Chairperson: K. Burridge, *University of North Carolina, Chapel Hill*

Cell-Cell Interactions
Chairperson: M. Takeichi, *Kyoto University, Japan*

Finale
Chairperson: E.R. Kandel, *HHMI, Columbia University, New York*

Summary: Melvin Simon, *California Institute of Technology, Pasadena*

B. Stillman, J. Kimble

Molecular Biology of Aging

April 22–April 26, 1992

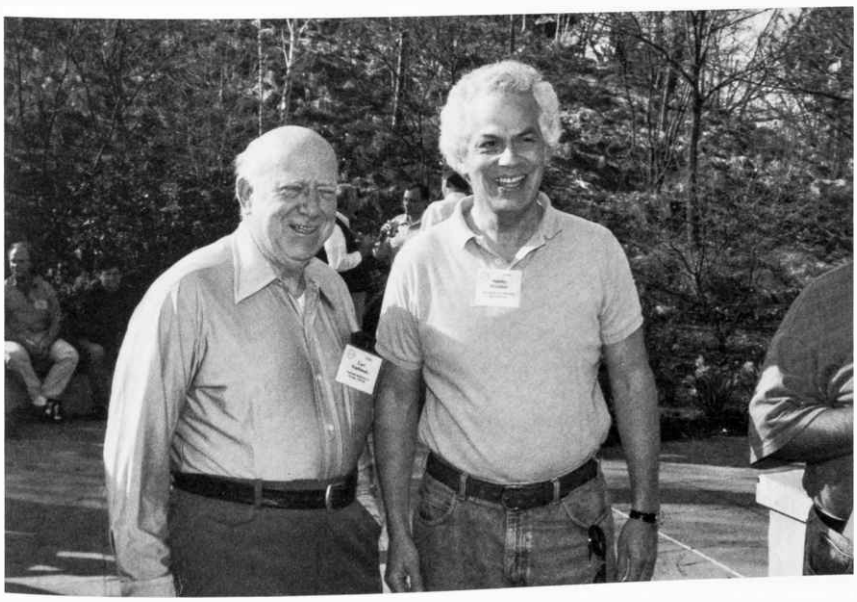
ARRANGED BY

Caleb Finch, University of Southern California, Los Angeles
Stanley Prusiner, University of California, San Francisco

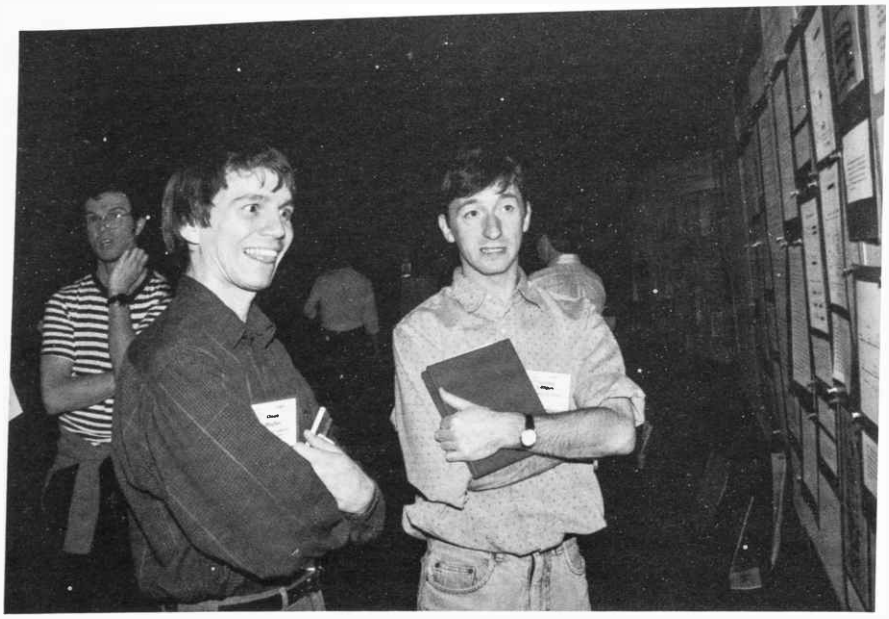
126 participants

This meeting was intended to interface several communities of researchers of the general theme of aging, as well as to highlight ongoing advances. Because the cell biologists and neurobiologists who work on aging, for example, have rarely attended the same meetings, there were overview lectures at the beginning and closing of the program. Among the cross-fertilizing themes was the recognition of the fact that abnormal proteins accumulate during normal aging, as well as in Alzheimer's disease. The theories of somatic cell genomic damage as a mechanism in aging are finally gaining credibility by demonstrations of loss of telomeric DNA and by mitochondrial DNA deletions. Although molecular approaches were represented throughout the presentations, the full powers of recombinant technology are just beginning to be applied to short-lived invertebrates. A major open question is the possibility of general molecular mechanisms responsible for aging changes, despite the manifest species specificity of many diverse pathological and physiological changes. Enthusiasm was high for further workshops and symposia in this general area.

This meeting was funded in part by the National Institute on Aging, Division of the National Institutes of Health.



E. Stadtman, S. Prusiner



O. Hughes, G. Lithgow

PROGRAM

Overviews
C.E. Finch, *University of Southern California*
G.M. Martin, *University of Washington*

Genome Stability
Chairperson: G.M. Martin, *University of Washington*

DNA Synthesis
Chairperson: V.J. Cristofalo, *Medical College of Pennsylvania*

Abnormal Proteins and Neurodegenerative Disease
Chairperson: E.R. Stadtman, *NHLBI/National Institutes of Health*

Neurodegenerative Diseases and Cell Death
Chairperson: M.L. Shelanski, *Columbia University*

Gene Expression
Chairperson: C.E. Finch, *University of Southern California*

Genetics of Neurodegeneration and Life Span
Chairperson: T.E. Johnson, *University of Colorado*

Closing Lectures and General Discussion
Chairpersons: D.L. Price, *Johns Hopkins University School of Medicine*
S.B. Prusiner, *University of California, San Francisco*

Neurobiological Basis of Epilepsy

April 29–May 2, 1992

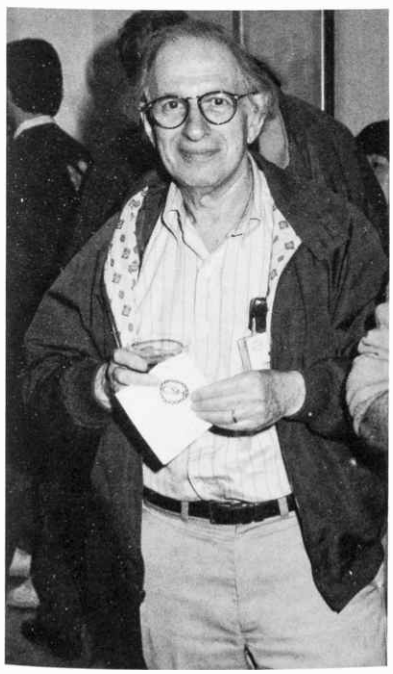
ARRANGED BY

Charles Gilbert, Rockefeller University
Fred Plum, Cornell University Medical College
Charles Stevens, The Salk Institute

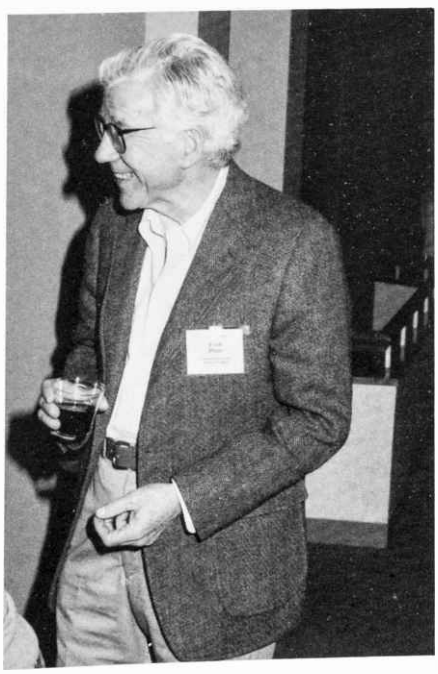
146 participants

This meeting provided an overview of current advances in neurobiology that may lead to an understanding of mechanisms underlying epilepsy. Given the considerable progress that has been made in elucidating the cellular mechanisms of neuronal excitability, it is anticipated that these advances will lead to an understanding of how seizures might occur in brains of patients with epilepsy. "The Neurobiological Basis of Epilepsy" meeting brought together clinicians and neuroscientists working in basic neuronal mechanisms. The clinicians described the clinical presentation of epilepsy, its neuropathology, and the pharmacology of anticonvulsants. The description of basic mechanisms included talks on synaptic mechanisms, neuronal circuits, gene expression and excitability, intracellular messengers, and development and plasticity. By bringing together molecular, cellular, systems and clinical approaches, the intention of the meeting was to apply advances in neuroscience to an understanding of the pathophysiology of epilepsy and to obtain insights into its treatment.

This meeting was funded by The Esther A. and Joseph Klingenstein Fund, Inc.



E. Kandel



F. Plum

PROGRAM

The Clinical Picture
Chairperson: F. Plum, *Cornell University Medical College*

Neuropathology of Epilepsy
Chairperson: F. Plum, *Cornell University Medical College*

Genetics and Epilepsy
Chairperson: D. Choi, *Washington University*

Anticonvulsants
Chairperson: C. Stevens, *The Salk Institute*

Synaptic Mechanisms
Chairperson: C. Stevens, *The Salk Institute*

Neuronal Circuits
Chairperson: C. Gilbert, *Rockefeller University*

Gene Expression and Excitability
Chairperson: C. Gilbert, *Rockefeller University*

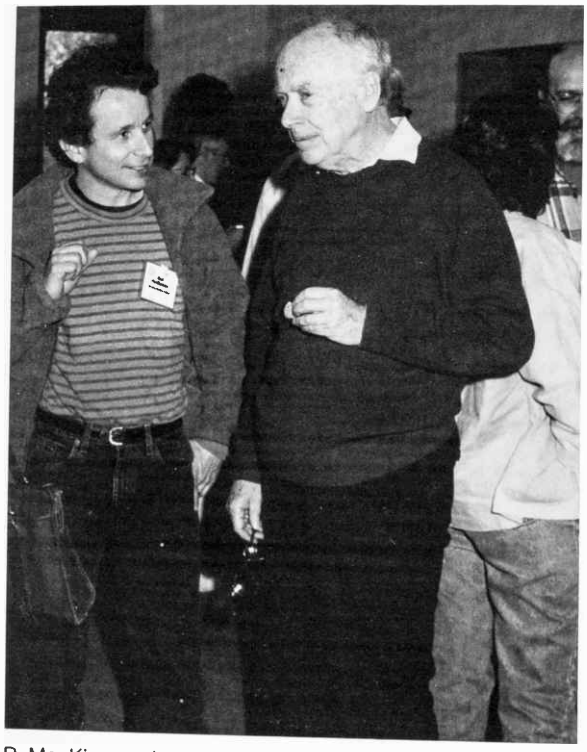
Intracellular Messengers
Chairperson: J. McNamara, *Duke University/Durham V. A. Medical Center*

Development and Plasticity
Chairperson: J. McNamara, *Duke University/Durham V. A. Medical Center*

Development and Plasticity (continued)
Chairperson: R. Dingledine, *University of North Carolina*

Summary
Chairperson: E. Kandel, *Columbia University*

Concert: Violinist: Scott Yoo, accompanied by Max Levinson, piano



R. MacKinnon, James Watson

Genome Mapping and Sequencing

May 6–May 10, 1992

ARRANGED BY

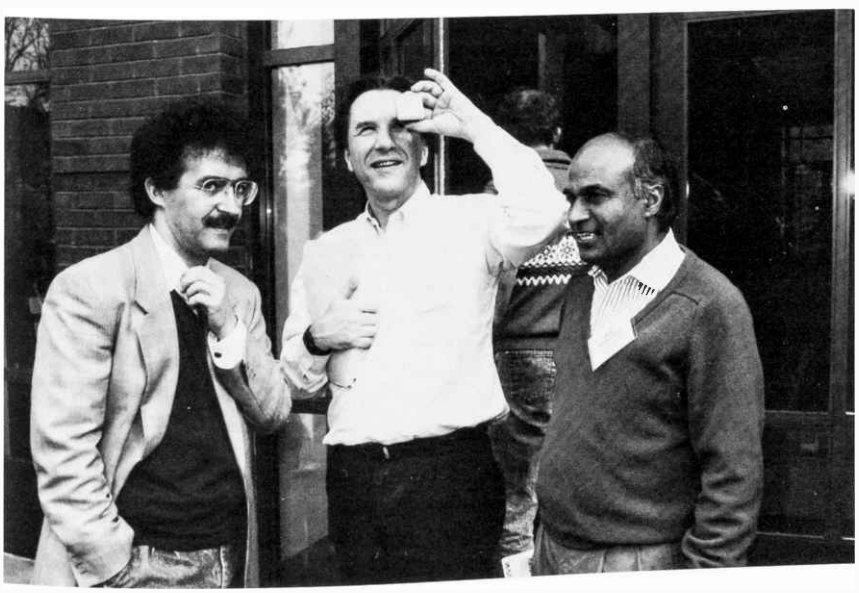
Richard Myers, University of California, San Francisco
David Porteous, Medical Research Council, Edinburgh, England
Richard Roberts, Cold Spring Harbor Laboratory

441 participants

The fifth Genome Mapping and Sequencing meeting reported on important progress in many areas over the past year. A recurrent theme was the synergism developing between genome studies of different organisms, from yeast to humans, via the nematode, molds, mice, and fruit flies. A major highlight was the impressive progress being made in large-scale genomic sequencing of *S. cerevisiae*, *C. elegans*, and even humans.

We heard how a European 35-center collaboration has just completed the sequencing of the entire yeast chromosome III. On this basis, the complete sequence of this organism seems now a distinct possibility within a few years. The current effort has revealed a multitude of new genes for genetic and functional analysis, the scale of the latter problem providing a major stimulus for new and exciting activity. Profound insights into the eukaryotic transcriptional map are sure to follow. The USA/UK collaboration to map and sequence *C. elegans* is already ahead of their target with 1 Mbp sequenced this year and 3 Mbp promised for 1993. With only modest improvements in efficiency, the 100-Mbp task could be completed this decade.

Sequencing the human genome poses altogether new problems of scale and complexity due to the high level of repeated DNA sequences, but again, the progress and insights into chromosomal and transcription organization, evolution,

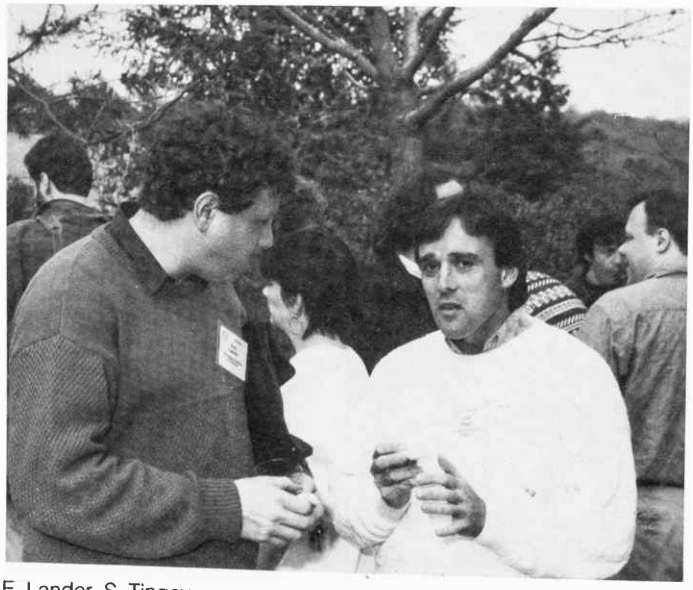


G.-J. Van Ommen, D. Cox, R. Kucherlapati

and polymorphic variation that have come from focused efforts to sequence gene complexes and random contiguous DNA tracts more than justifies the efforts. The alternative approach of cDNA cloning and sequencing has also made impressive progress, with several thousand new genes identified in *C. elegans*, humans, and mice. Will we need to extend each to full-length sequence for the true function to be predicted, or understood? Do biological experiments hold the key or can sequence comparisons provide at least some of the answers that this wealth of new data promises? Certainly, improved informatics is an essential complement to these large-scale genome analyses, and here too, we were provided with graphic evidence for important progress. Potential transcription units can be identified directly from raw sequences with remarkable confidence. Again, to keep pace with the demand for faster and longer sequencing capacity, there is clearly room for technical improvement and a need for further economy. There is still potential for very significant improvement of conventional technology, but we were also treated to a possible glimpse of the future, with sequencing by hybridization, mass spectroscopy, and microchip technology vying to make the quantum technological breakthrough.

High-density physical and genetic index maps are nearing completion in humans and mice. We heard too of the exciting progress being made toward complete cloned contig maps of several human chromosomes. The 40 Mbp of euchromatic Y is essentially cloned and ordered, a task completed in determined fashion by three pairs of hands in less than 2 years. For the autosome maps, everyone is looking for help from the remarkable YAC clone resources established at CEPH. They estimate that a quarter of the human genome is already in contigs and that 90% are 5 Mbp or larger. This is just what is needed as a base resource for positional cloning of disease genes.

The meeting maintained its reputation as the forum for innovative ideas and enabling strategies, this year featuring novel in situ hybridization techniques, mutation/polymorphism detection assays, and a variety of methods for introducing or creating novel restriction cleavage specificities, among others. Several ef-



E. Lander, S. Tingey

D. Patterson

fective methods for integrating complex genomic sources with cDNAs to isolate the transcribed components were described, filling an important gap in the positional cloning strategy for isolating candidate disease genes.

Finally, the warmest reception at the meeting was for Jim Watson, who more than anyone else has nurtured the Genome Project to the healthy and exciting state that it currently enjoys and which this meeting aims to reflect. We all look forward in anticipation to next year's meeting and trust to benefit from Jim Watson's continuing critical enthusiasm and vision for what the future goals must be.

Rich Roberts (with Maynard Olson and Charles Cantor) has coorganized this meeting from its inception, but after 5 years will bow out, allowing Bob Waterston to join Rick Myers and David Porteous in the largely pleasant task of organizing next year's meeting, with further assistance from Eric Green, Ray Gesteland, Barbara Trask, Kay Davies, and Rodney Rothstein.

This meeting was funded by the National Center for Human Genome Research, National Institutes of Health.

PROGRAM

Human Maps and Diseases
Chairperson: U. Francke, *Stanford University, California*

Methods: Cloning and Automation
Chairperson: W. Szybalski, *University of Wisconsin, Madison*

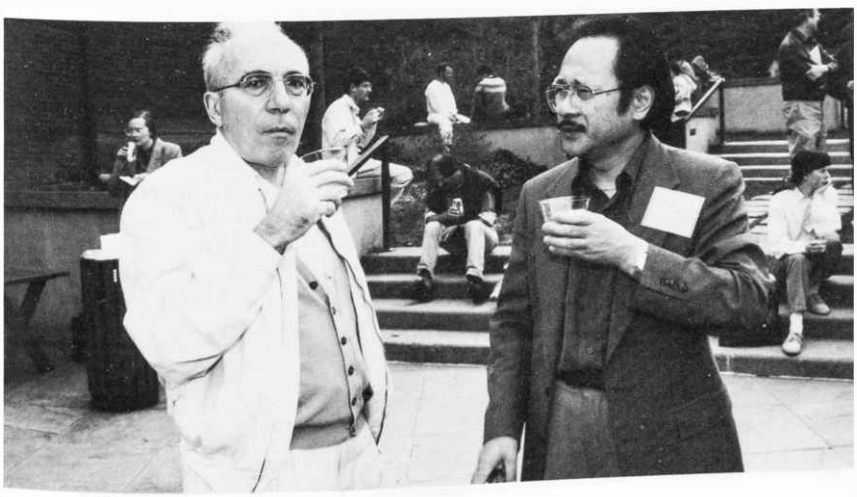
Sequencing Methodology
Chairperson: R. Gesteland, *University of Utah, Salt Lake City*

Methods: Mapping
Chairperson: B. Trask, *Lawrence Livermore National Laboratory*

Informatics
Chairperson: R. Myers, *University of California, San Francisco*

Large-scale Sequence Projects
Chairperson: D. Porteous, *MRC, Human Genetics Unit, Edinburgh*

Maps of Model Organisms
Chairperson: G. Miklos, *Australian National University, Canberra*



G. Bernardi, N. Shimizu

The Cell Cycle

May 13–May 17, 1992

ARRANGED BY

David Beach, Cold Spring Harbor Laboratory
Arnold Levine, Princeton University
Joan Ruderman, Harvard Medical School

338 participants

Scientists from research centers in Europe, the Far East, and the United States gathered to discuss their latest research concerning control of the cell cycle and cell proliferation. The program included 74 speakers who discussed such fundamental problems as control of mitosis; entry into the cell cycle; the role of anti-oncogenes and tumor suppressor genes in regulating cell growth; DNA replication; cell differentiation and cessation of proliferation; role of oncogenes and growth factors in control of cell proliferation; and chromosome behavior and division. The participating leading researchers in these areas of research use a variety of approaches from genetics to biochemistry to study control of the cell cycle. A variety of model systems from yeast to humans were discussed, and the meeting provided the opportunity to demonstrate how these different approaches complement each other. The meeting was one of great intensity and scientific interchange.

Contributions from the Corporate Sponsors provided core support for this meeting.

PROGRAM

START
Chairperson: A.J. Levine, *Princeton University*

Networking I
Chairperson: J.V. Ruderman, *Harvard Medical School*

Networking II
Chairperson: D. Beach, HHMI, *Cold Spring Harbor Laboratory*

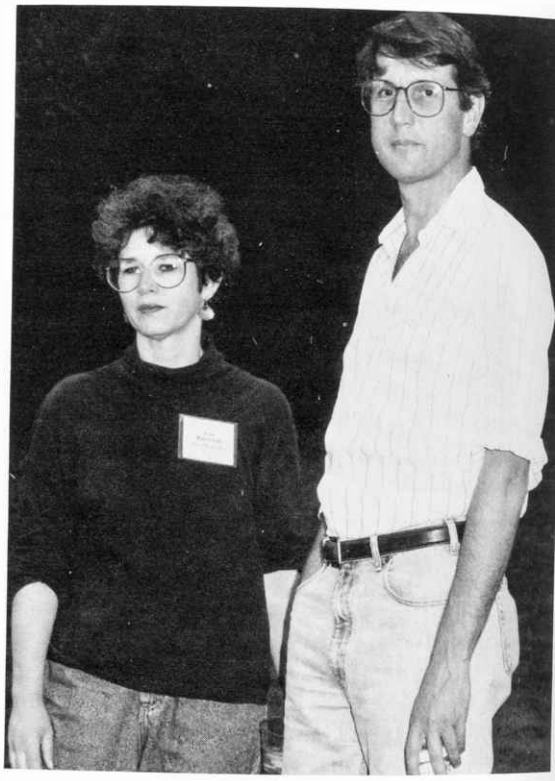
DNA Synthesis
Chairperson: H. Piwnica-Worms, *Tufts University*

CDC2 Activation
Chairperson: J.L. Campbell, *California Institute of Technology*

Signaling
Chairperson: B. Futcher, *Cold Spring Harbor Laboratory*

Checkpoints
Chairperson: L. Breeden, *Fred Hutchinson Cancer Research Center*

Phosphatases Across the Cycle
Chairperson: P.H. O'Farrell, *University of California, San Francisco*



J. Rudderman, D. Beach

RNA Tumor Viruses

May 19–May 24, 1992

ARRANGED BY

Eric Hunter, University of Alabama, Birmingham
Volker Vogt, Cornell University

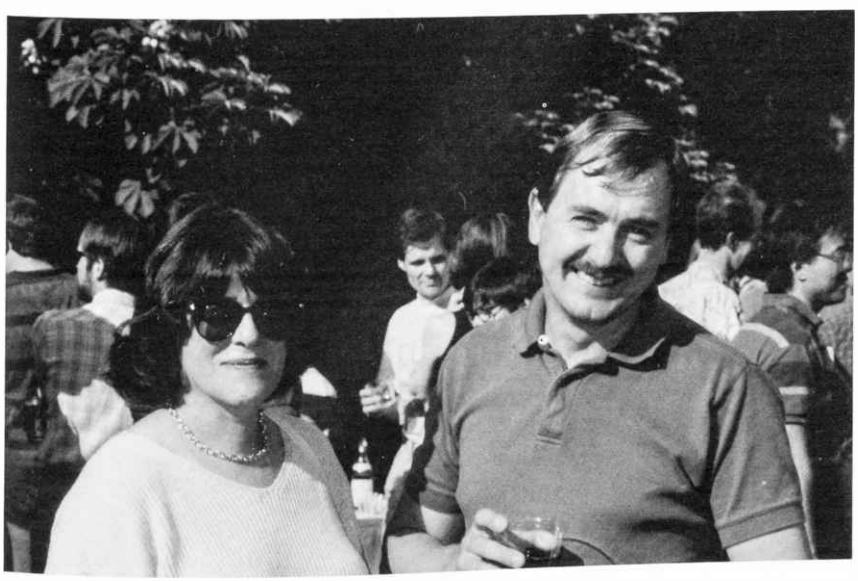
411 participants

The 1992 RNA Tumor Virus meeting featured papers covering a broad range of topics in retrovirus biology, including virus-host interactions, virus replication mechanisms, and viral pathogenesis, the latter including both oncogenesis and immunosuppression. A strength of the meeting as always was the diversity of retroviruses included in the various scientific sessions. This allowed the increasing number of presentations on pathogenic human retroviruses to be viewed in the broad perspective of the retrovirus field as a whole.

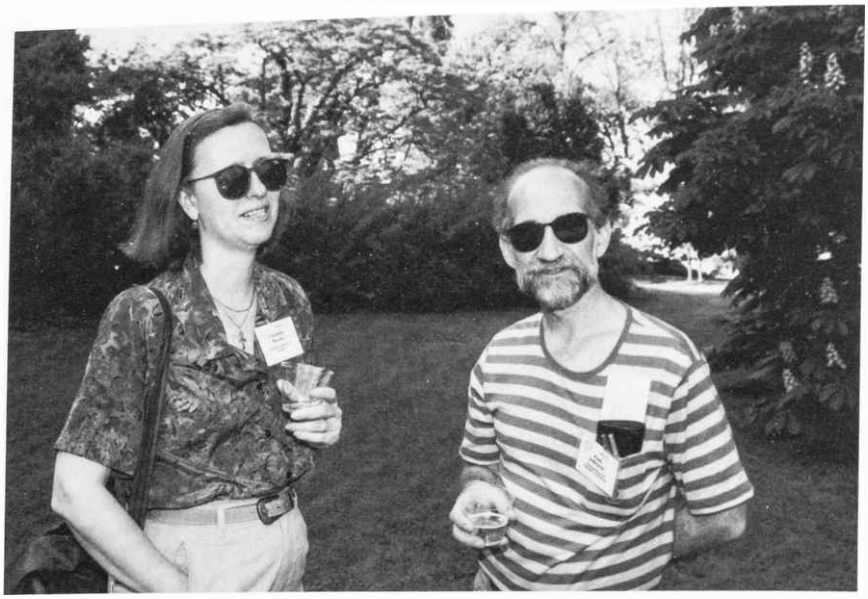
This year's meeting was characterized by renewed interest in the processes of virus assembly, virus entry, and reverse transcription/integration, which together comprised more than half the papers presented. The interplay of viral and host factors in the regulation of retroviral transcription and posttranscriptional processes was a major topic of discussions, and the role of "nonessential" genes in viral pathogenesis was reemphasized.

This was a historical and "final" CSH RNA Virus Meeting, since it was felt by a majority of the participants that the term RNA Tumor Virus was anachronistic, particularly when one considers the numbers of papers dealing with nononcogenic retroviruses and retrotransposons. The 1993 meeting will therefore bear the title "CSH Retroviruses Meeting."

Contributions from the Corporate Sponsors provided core support for this meeting.



T. Grodzicker, E. Hunter



C. Kozak, P. Jolicoeur

A. Pinter, J. Cunningham

PROGRAM

Virus Assembly
Chairpersons: E. Barklis, *Oregon Health Sciences University*
J. Wills, *Penn State University School of Medicine*

Encapsidation and Maturation
Chairpersons: C. Carter, *State University of New York, Stony Brook*
K. Strebel, *NIAID, National Institutes of Health*

Virus Entry
Chairpersons: J. Cunningham, *HHMI and Harvard Medical School*
A. Pinter, *Public Health Research Institute, New York*

Reverse Transcription
Chairpersons: F. Clavel, *Institute Pasteur, Paris*
S. Hughes, *National Cancer Institute-FCRDC, Frederick*

Integration and Recombination
Chairpersons: R. Craigie, *NIDDK, National Institutes of Health*
R. Katz, *Fox Chase Cancer Center, Philadelphia*

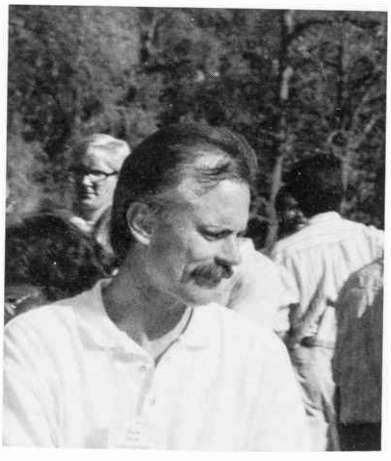
Expression 1: Transcription
Chairpersons: L. Ratner, *Washington University School of Medicine*
N. Speck, *Dartmouth Medical School*

Expression 2: Posttranscription
Chairpersons: K. Beemon, *Johns Hopkins University*
E.-T. Jeang, *NIAID, National Institutes of Health*

Pathogenesis
Chairpersons: R. Desrosiers, *New England Regional Primate Research Center*
H. Fan, *University of California, Irvine*

Retrovirus Potpourri
Chairpersons: J. Elder, *Scripps Research Institute*
P. Jolicoeur, *Clinical Research Institute of Montreal*

Expression 3: *trans*-Activation
Chairpersons: D. Derse, *National Cancer Institute-FCRDC, Frederick*
B. Felber, *National Cancer Institute-FCRDC, Frederick*



D. Derse

Molecular Biology of SV40, Polyoma, and Adenoviruses

August 12–August 16, 1992

ARRANGED BY

Terri Grodzicker, Cold Spring Harbor Laboratory
David Livingston, Dana-Farber Cancer Institute
Carol Prives, Columbia University

234 participants

The small DNA tumor viruses—SV40, polyoma, and adenoviruse—have long served as model systems to study cellular transcription, replication, and control of cell growth. Approximately 250 scientists gathered at the 1992 meeting to present their latest results in about 200 talks and posters.

Much of the emphasis at the meeting concerned the interaction of viral regulatory proteins (e.g., adenovirus E1A proteins, SV40 T antigen) with cellular proteins such as p53, the retinoblastoma (*RB*) gene product, and cyclins, as well as cell-cycle changes in protein complexes and their state of phosphorylation. The role of protein modifications and interactions of viral regulatory proteins with cellular kinases and phosphatases also received attention. Several talks described the role of E1A in induction of apoptosis and that of the E1A 19K proteins in suppression of apoptosis. Much research continues to focus on the purification and analysis of cellular proteins involved in viral DNA replication and the use of in vitro systems to dissect the replication process. The study of transcriptional regulation and the interactions of viral E1A proteins and T antigens(s) with components of the transcriptional apparatus using in vitro as well as in vivo systems was extensively discussed. Talks were also given on RNA splicing, control of poly(A)-site usage, translational control, cell-cycle-specific protein modifications,

D. Livingston, M. Green

and the role of different viral proteins in the host's immune response. The meeting proved a highly profitable occasion for the discussion of new results emerging from the study of viral proteins and virus-host cell interactions.

Contributions from the Corporate Sponsors provided core support for this meeting.

PROGRAM

Oncogenes and Transcriptional Activation
Chairperson: M.R. Green, *University of Massachusetts Medical Center, Worcester*

Protein Complexes
Chairperson: D. Livingston, *Dana-Farber Cancer Institute, Boston, Massachusetts*

DNA Replication
Chairperson: B. Stillman, *Cold Spring Harbor Laboratory*

Transcription: Adenoviruses
Chairperson: A. Berk, *University of California, Los Angeles*

Transformation
Chairperson: E. Fanning, *Universitat Munchen, Germany*

Transcription: SV40, Polyoma
Chairperson: C. Cole, *Dartmouth Medical School, Hanover, New Hampshire*

Posttranscription
Chairperson: J. Mertz, *University of Wisconsin, Madison*

Host-Virus Interactions
Chairperson: E. White, *Rutgers University, Piscataway, New Jersey*

M. Botchan, M. Fried, T. Grodzicker

C. Prives, J. Alwine, B. Stillman

Molecular Genetics of Bacteria and Phages

August 18–August 23, 1992

ARRANGED BY

Susan Gottesman, National Cancer Institute
Peter Model, Rockefeller University
Miriam Susskind, University of Southern California

210 participants

More than 200 people attended the 1992 phage meeting at Cold Spring Harbor Laboratory, and 169 abstracts were presented in eight platform sessions and two poster sessions. Thirty six percent of the abstracts were submitted from outside the United States, and participation in the meeting continues to be an excellent mix of scientists at a variety of levels in their scientific careers from all over the world.

As in the past, the meeting emphasized the basic biology of bacterial systems and responses to environmental change, including interactions between phage and their hosts. Subject matter presented covered a wide range, including regulation of gene expression, studies on entry and exit from the cell, replication and recombination, and protein structure. Gourse and co-workers extended earlier observations by other investigators on the importance of the RNA polymerase α subunit in positive activation to a role for this subunit in factor-independent activation of rRNA promoters, suggesting changes in both our understanding of positive activation and the definition of a promoter. Deho and co-workers reported that the immunity determinant for the P4 bacteriophage is a short RNA, which appears to act by promoting premature termination of another P4 tran-

J. Watson, J. Roberts, S. Gottesman

script. Work on the lysis protein of phage ϕX174 by Schrot and co-workers in Austria suggests that this protein causes fusion between inner and outer membranes, providing a direct route out of the cell for the phage.

Contributions from the Corporate Sponsors provided core support for this meeting.

PROGRAM

RNAP-Promoter Interactions
Chairperson: W. McAllister, *State University of New York, Brooklyn*

Transcription: Promoter Clearance, Elongation, and Termination
Chairperson: R. Weisberg, *National Institutes of Health*

Repressors and Activators
Chairperson: M. Howe, *University of Tennessee*

Antisense and Translational Control
Chairperson: A. Poteete, *University of Massachusetts*

Regulatory Circuits
Chairperson: R. Calendar, *University of California, Berkeley*

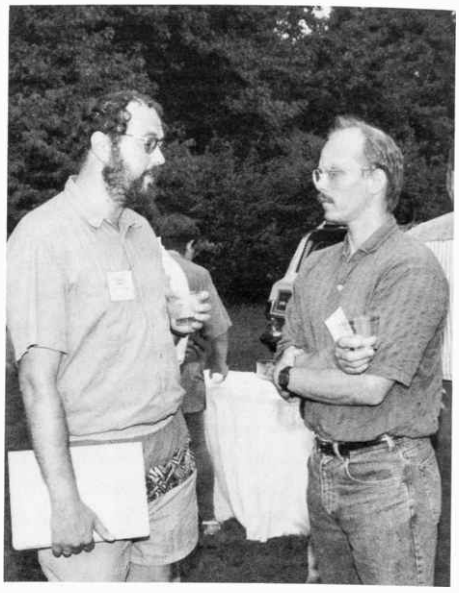
Protein Structure, Chaperonins, and Heat Shock
Chairperson: R. Ebright, *Rutgers University*

The Outer Limits
Chairperson: M. Russel, *Rockefeller University*

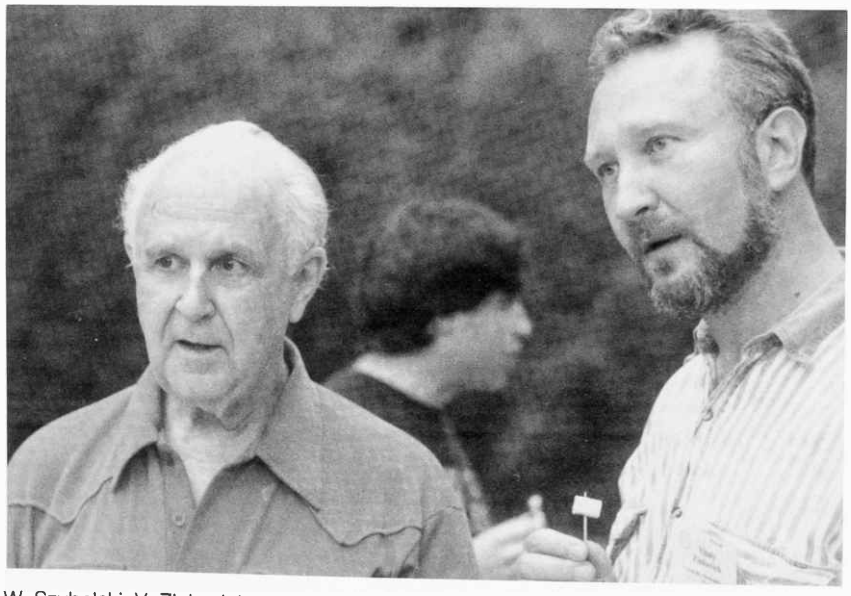
DNA Replication and Partitioning
Chairperson: M. Yarmolinsky, *National Institutes of Health*

RNA Processing, Intron Homing, and Recombination
Chairperson: D. Shub, *State University of New York, Albany*

Genome Analysis and Rearrangement
Chairperson: N. Craig, *Johns Hopkins University*



H. Bahl, B. Bukau



W. Szybalski, V. Zinkevich

Mouse Molecular Genetics

August 26–August 30, 1992

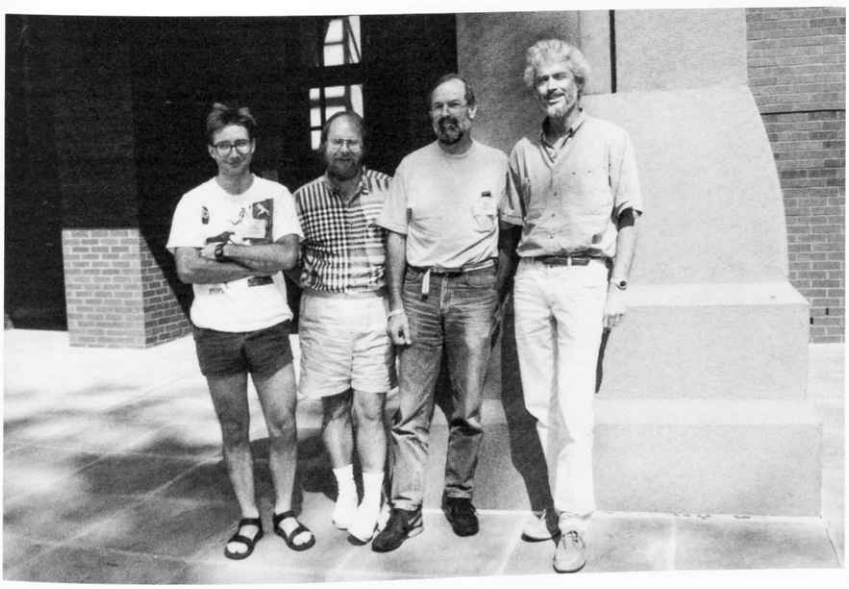
ARRANGED BY

Anton Berns, The Netherlands Cancer Institute
Douglas Hanahan, University of California, San Francisco
Robb Krumlauf, MRC National Institute for Medical Research
Andy McMahon, Roche Institute of Molecular Biology

424 participants

The sophisticated approaches currently available to the mouse molecular geneticist, which allow gene expression to be precisely manipulated in vivo, are revolutionizing our ability to probe the complex mechanisms involved in generating a normal adult mouse. The clear spin-off from this work is a better understanding of the normal workings of humans, and the development of rational approaches toward the molecular elucidation and eventual treatment of human disease. The annual Mouse Molecular Genetics meeting, held this year at Cold Spring Harbor Laboratory, was a landmark meeting with important advances reported in the broad fields of developmental biology, immunology, and oncology.

At the previous meeting held in Heidelberg in 1991, the organizers hit upon a meeting format that was successfully adopted at Cold Spring Harbor Laboratory. Session chairs were invited for each session. In addition, one prominent researcher was invited to give the opening talk in each session. All other speakers (eight per session) were selected from the list of submitted abstracts. Thus, this meeting was mainly a forum for the advanced graduate student and postdoctoral fellow to present their own work, under the guidance of an experienced chairperson, an opportunity that is regrettably rare today. So great was the interest in this



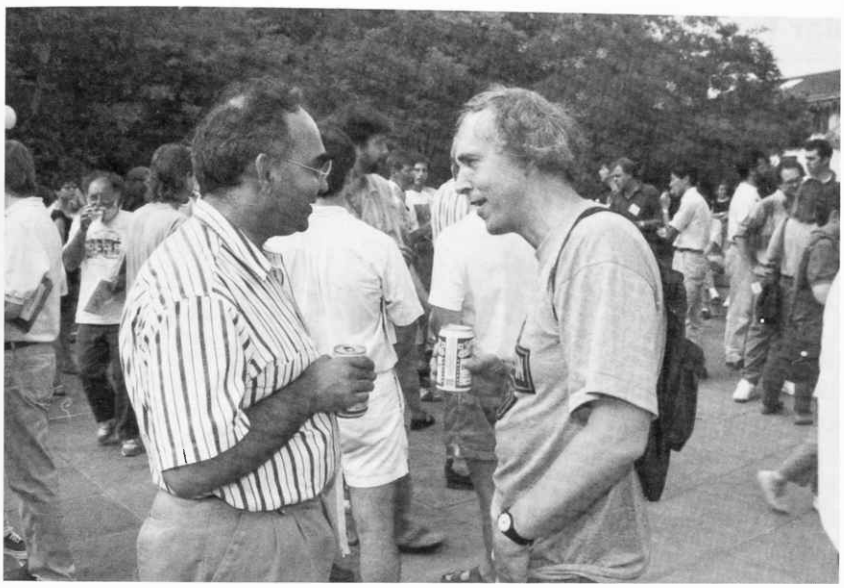
A. McMahon, R. Krumlauf, A. Berns, D. Hanahan

meeting that it was difficult to choose only 64 platform presentations from among the many excellent abstracts. Fortunately, two well-attended poster sessions allowed the presentation of 150 posters. As usual, Cold Spring Harbor proved to be an exceptional environment for informal interactions, perhaps the most important part of any meeting.

The most remarkable aspect of the meeting was the dramatic realization of the efficient predictable targeting of mouse genes. No less than 27 talks dealt with novel mouse mutants generated by targeted mutagenesis in embryo stem cells, arguably the most important advance in mouse genetics in the last decade. Although it is hard to select only a few highlights from the many presentations in which important advances were made using this approach, some of the principal findings are described below.

Several groups reported the targeting of tumor suppressor genes, in particular, the retinoblastoma gene, which resulted in a heightened tumor susceptibility. Surprisingly, targeting of a peptide growth factor, α-inhibin, suggests that this protein is normally involved in gonadal growth control and thus acts as a tumor suppressor. Together, these mice offer new ways in which the development of tumors and the use of therapeutic agents can be explored. Disease models of cystic fibrosis, hypercholesterolemia, osteoarthritis, and ulcerative colitis were generated by mutating the cystic fibrosis transmembrane conductance regulator, apolipoprotein E, collagen IX, and IL-2 genes, respectively. In the field of immunology, an impressive array of proteins associated with antigen presentation and recognition, including T-cell receptors, MHC class I and II molecules, and peptide transporters, have been disrupted, in many cases validating current models, but also generating some unexpected findings. In the area of developmental biology, convincing evidence is finally emerging linking homeobox gene expression with skeletal patterning along the anterior-posterior axis. Study of organ development also received a boost from the demonstration that a proto-oncogene receptor tyrosine kinase, c-ret, is essential for some aspect of the inductive interaction involved in early kidney development.

P. Rowe, E. Fuchs



I. Kola, A. Dunn

It seems likely that interest in the use of mice as experimental animals will grow. This will require improved mechanisms for distributing materials, a topic that led to a lively discussion in a packed auditorium. Clearly, the mouse will remain in the vanguard of genetic research for the foreseeable future, funding permitting. We are grateful to Dr. Doug Hanahan who stepped down this year after many years of organizing this meeting.

This meeting was funded in part by the National Science Foundation, and the following divisions of the National Institutes of Health: the National Institute of Child Health and Human Developmnent, the National Cancer Institute, and the National Heart, Lung and Blood Institute.

PROGRAM

Transcriptional Regulation in Development
Chairperson: S. Tilghman, *Princeton University, New Jersey*

Pattern Formation in Early Development
Chairperson: J. Rossant, *Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada*

Organ Development and Differentiation
Chairperson: B. Hogan, *Vanderbilt University Medical School, Nashville, Tennessee*

Hematopoiesis
Chairperson: I. Weissman, *Stanford University Medical Center*

Mouse Models of Disease
Chairperson: E. Wagner, *Institute for Medical Pathology, Vienna, Austria*

Mechanisms of Tumorigenesis
Chairperson: H. Varmus, *University of California, San Francisco*

New Technologies
Chairperson: R. Palmiter, *HHMI, University of Washington, Seattle*

Signals and Receptors
Chairperson: R. Jaenisch, *Whitehead Institute for Biomedical Research, Cambridge, Massachusetts*

Genetics and Molecular Biology of Breast Cancer

September 2–September 6, 1992

M.-C. King

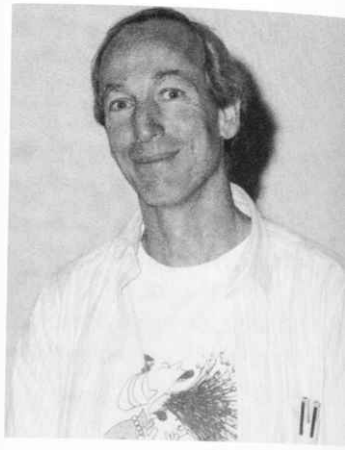
ARRANGED BY

Mary-Claire King, University of California, Berkeley
Marc Lippman, Georgetown University Medical Center

167 participants

In the past year, considerable progress has been made in understanding the genetics and molecular biology of breast cancer. Genes have been identified whose alteration, amplification, or loss in tumors is associated with invasion, progression, or metastasis. A gene has been mapped, and is likely to be cloned soon, that is responsible for inherited early-onset breast cancer and ovarian cancer and that appears also to be altered somatically in breast and ovarian tumors. The relationships among growth factors and receptors active in normal and malignant breast tissue have been more precisely defined. The goal of this meeting was to bring together investigators working with genes, with growth factors and their receptors, and with breast cancer clinically in order to identify how and when these three avenues of research will converge.

This meeting was funded in part by the U.S. Dept. of Energy and the National Cancer Institute, National Institutes of Health.



M. Lippman

PROGRAM

Welcoming Remarks: J.D. Watson

Plenary Session
Chairperson: J.D. Watson, *Cold Spring Harbor Laboratory*

Current Clinical Realities, Epidemiology, and Pathology
Chairperson: I.C. Henderson, *University of California, San Francisco*

Genetics I
Chairperson: M.-C. King, *University of California, Berkeley*

Genetics II
Chairperson: M.-C. King, *University of California, Berkeley*

Growth Factors and Their Receptors I
Chairperson: M. Lippman, *Georgetown University Medical Center*

Growth Factors and Their Receptors II
Chairperson: M. Lippman, *Georgetown University Medical Center*

Invasion and Metastasis
Chairperson: S.A. Aaronson, *NCI, National Institutes of Health*

I.C. Henderson

Translational Control

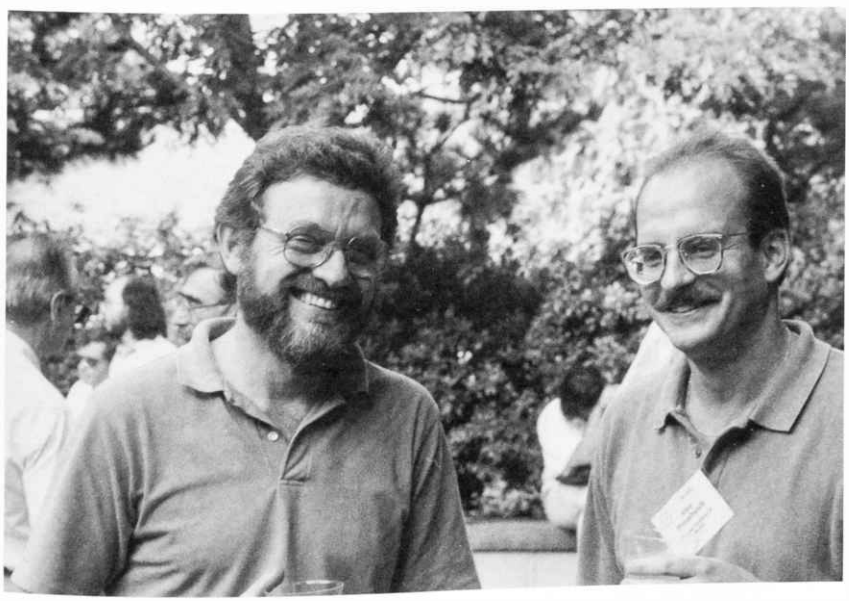
September 9–September 13, 1992

ARRANGED BY

Alan Hinnebusch, National Institutes of Health
Michael Mathews, Cold Spring Harbor Laboratory

377 participants

The Translational Control meeting held at Cold Spring Harbor Laboratory this year exceeded the previous two in the number of participants and abstracts communicated. As in the past, the research involved many different organisms, eukaryotic and prokaryotic, and diverse regulatory mechanisms. It is clear that translational control plays an important role in a variety of biological phenomena, including responses to nutrient availability, environmental stress, and virus infection, the control of cell proliferation, and development. Regulation occurs at the level of mRNA stability, translation initiation, elongation, or termination. A novel means of regulating initiation was described in bacteria where ribosomes become trapped on the mRNA in nonproductive complexes. In eukaryotes, the 3′ UTR was implicated in various mechanisms affecting mRNA stability or translational efficiency. Small open reading frames (ORFs) were shown to impede the flow of scanning ribosomes by a mechanism involving the ORF-encoded peptides. Internal initiation of translation, first identified in picornaviruses, surfaced in several cellular mRNAs, and there appeared to be considerable diversity in the RNA elements and *trans*-acting factors that mediate these specialized initiation events. Regulation of initiation factor activity by phosphorylation continued to be the focus of much research, especially for the eIF-2 and eIF-4E that function in regulating cellular proliferation. The mammalian interferon-induced eIF-2α kinase



M. Mathews, A. Hinnebusch

is targeted by many viruses, and the molecular mechanisms employed by regulators from adenovirus and vaccinia virus are coming into focus. An eIF-2α kinase from yeast, along with subunits of the GDP-GTP exchange factor for eIF-2, was implicated in gene-specific translational control. Other studies with yeast led to the identification of new initiation factors not previously detected in mammalian cell-free translation systems. The rapid progress witnessed at this meeting is clearly attributable to the powerful combination of genetic, molecular, and biochemical techniques being applied to the study of translational control in diverse organisms.

This meeting was funded in its entirety by ICN Biomedicals, Inc.

PROGRAM

Initiation: IREs and IRESs
Chairperson: M. G. Katze, *University of Washington*

Prokaryotes
Chairperson: D.A. Steege, *Duke University Medical Center*

Growth Control and Development
Chairperson: C.G. Proud, *University of Bristol, United Kingdom*

RNA-Protein Interactions and mRNA Stability
Chairperson: J.B. Harford, *National Institutes of Health*

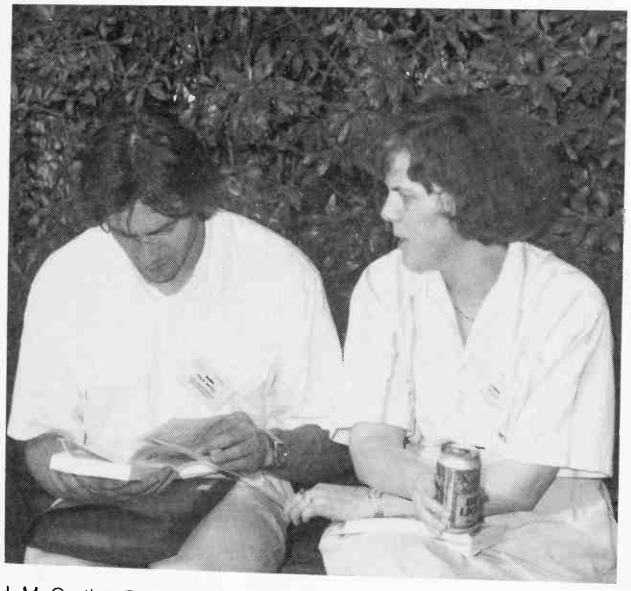
Elongation and Termination
Chairperson: R. Weiss, *University of Utah*

Viral Systems
Chairperson: R.J. Schneider, *New York University Medical Center*

Factors and Mechanism
Chairperson: T.F. Donahue, *Indiana University*

Concert: Mikhail Yanovitsky, Pianist

Initiation: ORFs and Kinases
Chairperson: R. Rhoads, *Louisiana State University Medical Center*



J. McCarthy, D. Steege

T. Donahue, B. Castilho-Valavicius

Modern Approaches to New Vaccines Including Prevention of AIDS

September 16–September 20, 1992

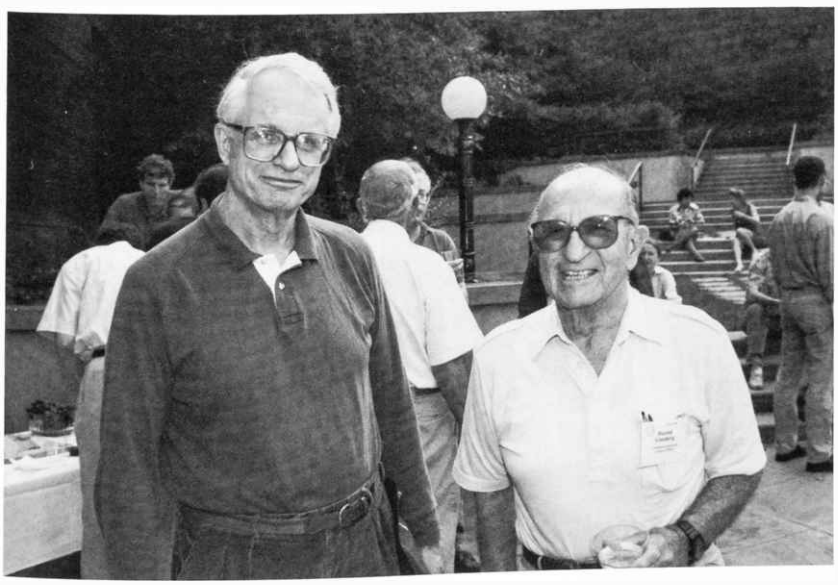
ARRANGED BY

Fred Brown, USDA, Plum Island Animal Disease Center
Robert M. Chanock, NIAID, National Institutes of Health
Harold S. Ginsberg, Columbia University College of Physicians & Surgeons
Richard A. Lerner, Research Institute of Scripps Clinic

214 participants

The tenth annual meeting on Modern Approaches to New Vaccines provided an opportunity for an unusually varied group of basic microbiologists, clinical investigators, epidemiologists, basic immunologists, and pharmaceutical industry scientists to interact and exchange their latest research observations concerning ways to improve immunoprophylaxis and extend its reach.

Many new advances were reported. Perhaps the most exciting new method for active immunoprophylaxis described at the meeting was a simple but effective form of direct genetic immunization. Several groups reported that direct inoculation of cloned DNA into muscle resulted in prolonged expression of the protective protein antigen encoded by the DNA. Animals immunized in this manner developed resistance to challenge infection. Also, it was reported that human antibody Fab fragments with high functional activity could be cloned from a recombinatorial library with high efficiency. The availability of such antibodies should provide new opportunities for passive immunotherapy and immunoprophylaxis because some of these monoclonal Fabs exhibit extremely high neutralizing activity for the virus against which the immunoglobulin fragments are directed.

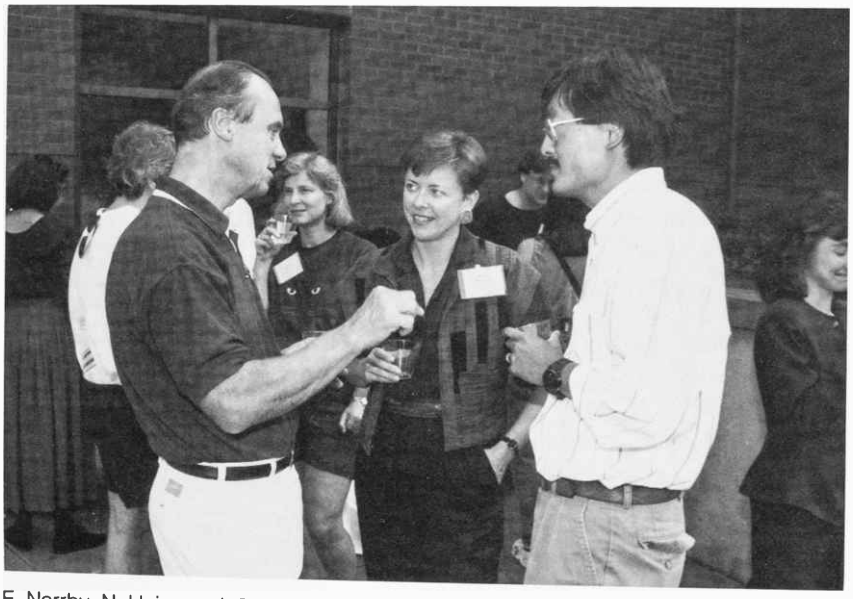


R. Chanock, H. Ginsberg

A potential breakthrough in immunization was described for the papillomaviruses, which are associated with a significant proportion of human cancers worldwide. A high level of papillomavirus neutralizing antibodies was induced for the first time by immunizing animals with a papillomavirus outer capsid protein expressed by a recombinant baculovirus. Recent advances in another active field of research suggests that nonsegmented negative-strand RNA viruses, as well as segmented double-stranded RNA viruses may soon be amenable to genetic manipulation at the cDNA level. Although rescue of these viruses in an infectious form from their cDNA has not yet been achieved, identification of *cis*-acting signals for virus transcription and replication has made it possible to rescue synthetic minigenomes from cDNA-encoded RNA transcripts using a helper-virus-dependent system.

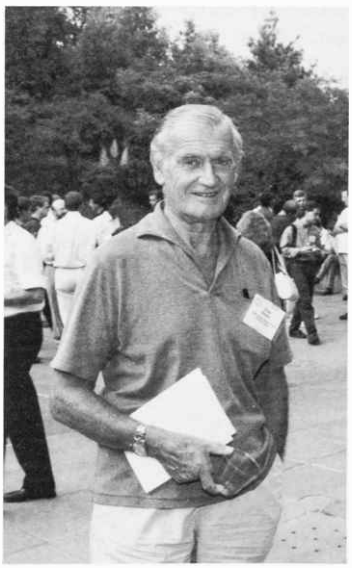
Many issues and developments in experimental immunoprophylaxis of AIDS were discussed during the course of the meeting. Perhaps most relevant were a series of presentations that addressed the mechanism responsible for the resistance to challenge exhibited by monkeys immunized with inactivated simian immunodeficiency virus (SIV) originally derived from human cell cultures. It now appears that sterile immunity against cell-free SIV challenge is due in many instances to antibodies directed against human host-cell components associated with vaccine virus rather than virus-specific immunity. Nonetheless, immunized monkeys that became infected following challenge with simian-cell-associated SIV did exhibit resistance to AIDS-like disease under conditions indicating that the protective effect was virus-specific. Finally, in one circumstance, sterile immunity to cell-associated SIV challenge was induced in 50% of monkeys immunized with inactivated SIV under conditions in which host-cell antigens did not appear to play a role. The last two observations offer some hope for the eventual control of AIDS by immunization.

Contributions from the Corporate Sponsors provided core support for this meeting.



E. Norrby, N. Haigwood, S.-L. Hu

B. Murphy, V. Hirsh, D. McPhee



F. Brown

PROGRAM

Immunology I
Chairperson: D. Burton, *Scripps Research Institute, La Jolla*
Overview Lecture: Dennis Burton, *Scripps Research Institute, La Jolla*

AIDS I
Chairperson: N.L. Letvin, *Harvard Medical School, New England Regional Primate Research Center*

Virology I
Chairperson: A. Kapikian, *NIAID, National Institutes of Health*

Immunology, Bacteriology, and Parasitology
Chairperson: F. Brown, *USDA, Plum Island Animal Disease Center*

AIDS II
Chairperson: E. Norrby, *Karolinska Institute, Stockholm*

Virology II
Chairperson: P. Collins, *NIAID, National Institutes of Health*

Aids III
Chairperson: H. Ginsberg, *Columbia University College of Physicians & Surgeons*

Bacteriology and Parasitology
Chairperson: B.R. Murphy, *NIAID, National Institutes of Health*

Aids IV
Chairperson: V.M. Hirsch, *NIAID, National Institutes of Health*
Summary: V.M. Hirsch, *NIAID, National Institutes of Health*

Gene Therapy

September 22–September 26, 1992

ARRANGED BY

W. French Anderson, National Institutes of Health, NHLBI
Theodore Friedmann, University of California, San Diego
Richard Mulligan, Massachusetts Institute of Technology

315 participants

The first Cold Spring Harbor meeting devoted to Human Gene Therapy was held on September 22–26, 1992. Since the initial discussions of the potentials for human gene therapy in the early 1970s, there has been enormous interest in this potentially powerful new form of medicine, and impressive technical advances during the past decade have now indicated that a genetic approach to the correction of many human ailments will become possible. Within the past several years, the first experimental gene therapy studies with human patients have been started with patients suffering both from genetic disorders and from acquired diseases such as cancer, AIDS, Parkinson's disease, Alzheimer's disease, and many others. Both the basic science aspects and the clinical applications of these advances were reviewed. This first Cold Spring Harbor meeting is widely seen as recognition of the importance of this conceptually new approach to therapy and to indicate broad acceptance of it by the scientific, medical, and policy communities. The development of human gene therapy is now in an explosive and productive stage of implementation, and Cold Spring Harbor will continue to serve as a major avenue for review and catalysis of the field by holding additional meetings devoted to gene therapy. The second meeting is to be held in the fall of 1994. This meeting was funded by The Wellcome Trust.

PROGRAM

Hematopoietic Cells
Chairperson: T. Friedmann, *University of California, San Diego*

Metabolic Disease I
Chairperson: I.M. Verma, *Salk Institute, La Jolla, California*

Cancer
Chairperson: R.C. Mulligan, *Massachusetts Institute of Technology*

CNS and Herpes Vectors
Chairperson: F.H. Gage, *University of California, San Diego*

Public Issues
Chairperson: N.A. Wivel, *National Institutes of Health*

Animal Models
Chairperson: F.W. Alt, *HHMI, Children's Hospital and Harvard Medical School*

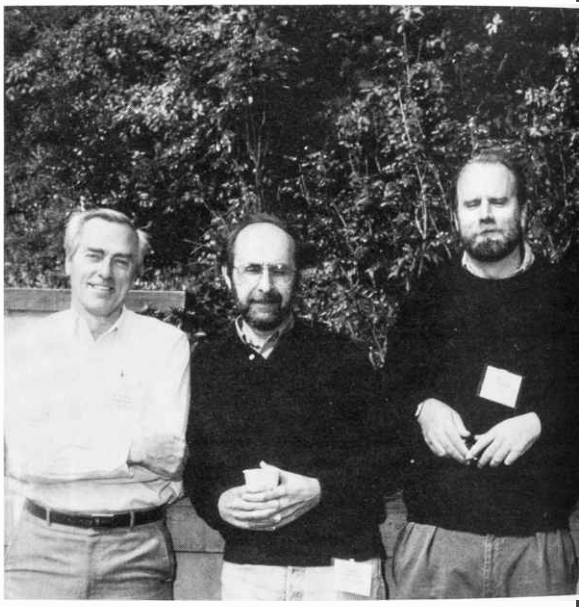
Metabolic Disease II
Chairperson: T. Friedmann, *University of California, San Diego*

Clinical Protocols
Chairperson: W.F. Anderson, *National Institutes of Health*

Clinical Overview: W.F. Anderson, *National Institutes of Health*

Gene Therapy for AIDs

Overview: R.A. Morgan, *National Institutes of Health*



W.F. Anderson, T. Friedmann, R. Mulligan

Learning and Memory

September 30–October 4, 1992

ARRANGED BY

Ron Davis, Cold Spring Harbor Laboratory
Eric Kandel, Columbia University College of Physicians & Surgeons
Larry Squire, University of California, San Diego

104 participants

The 1992 Cold Spring Harbor meeting on Learning and Memory drew together many major practitioners in the subject area. The scope of the meeting was wide. It extended from studies typically placed within the field of cognitive psychology, to developmental aspects of the brain in relation to plasticity, to electrophysiological studies of synapses and synaptic plasticity, to the cellular and genetic analysis of learning typically dominated by invertebrate preparations, such as *Aplysia* and *Drosophila*. The wide scope provided one of the few opportunities for scientists applying molecular approaches to learning/memory to interact with, and discuss the problem with, those working at the systems level. With the success of this first meeting, the meeting is now planned to be held every other year.

Highlights presented at the meeting included the analysis of fear learning and its mediation by the amygdala, the clarification of the role of the prefrontal cortex in working memory, and an exhilarating session on long-term potentiation. In addition, the meeting included two talks on learning in a new experimental system, *C. elegans*.

This meeting was funded by the Marie Robertson Memorial Fund.

PROGRAM

Introductory Comments: R. Davis, *Cold Spring Harbor Laboratory*

Brain Systems and Memory
Chairperson: R.F. Thompson, *University of Southern California, Los Angeles*

Brain Plasticity, Learning, and Development
Chairperson: L.R. Squire, *Veterans Administration Medical Center and University of California, San Diego*

Cellular and Molecular Aspects of Synaptic Plasticity
Chairperson: R. Davis, *Cold Spring Harbor Laboratory*

Long-term Potentiation
Chairperson: P. Andersen, *Institute of Basic Medical Sciences, University of Oslo, Norway*

Cerebellar Learning, and Long-term Depression
Chairperson: R.W. Tsien, *Stanford University*

Innvertebrate Learning and Plasticity I
Chairperson: R.D. Hawkins, *HHMI, Columbia University*

Invertebrate Learning and Plasticity II
Chairperson: E.R. Kandel, *HHMI, Columbia University College of Physicians & Surgeons*

E. Kandel, R. Davis, L. Squire

EDUCATIONAL ACTIVITIES

POSTGRADUATE COURSES

The summer program of Postgraduate Courses at Cold Spring Harbor Laboratory is aimed at meeting the special need for training in interdisciplinary subjects that are either so new or so specialized that they are not adequately treated by universities. Our aim is to provide intensive study in the most recent developments and techniques in these subjects and to prepare students to enter directly into research in a particular area. To ensure up-to-date coverage of current research work, we bring together a workshop staff from many laboratories around the world and supplement this staff with a series of seminar speakers.

Cloning and Analysis of Large DNA Molecules

April 2–April 16

INSTRUCTORS

Birren, Bruce, Ph.D., California Institute of Technology, Pasadena
McCormick, Mary Kay, Ph.D., Los Alamos National Laboratory, New Mexico
Shepherd, Nancy, Ph.D., DuPont Merck Pharmaceutical Co., Wilmington, Delaware

ASSISTANTS

Munk, Chris, Los Alamos National Laboratory, New Mexico
Pfrogner, Beverly, DuPont Merck Pharmaceutical Co., Wilmington, Delaware
Saunders, Liz, Los Alamos National Laboratory, New Mexico
Wrestler, Janet, University of North Carolina, Chapel Hill

This course covered the theory and practice of manipulating and cloning high-molecular-weight DNA. Lectures and laboratory work dealt with the use of bacteriophage P1 and yeast artificial chromosome (YAC) cloning systems, the isolation and manipulation of high-molecular-weight DNA from mammalian cells for cloning (including the size-selection of >200-kb DNA fragments), and the analysis of high-molecular-weight DNA by pulsed-field gel (PFG) separation techniques. P1 and YAC recombinant DNA molecules were produced, introduced into cells (*E. coli* and yeast, respectively), and reisolated after appropriate clone selection and colony screening procedures. A variety of size standards for pulsed-field gel electrophoresis were prepared, and gels were run to compare the DNA separation capabilities of the common PFG techniques. Students gained experience with physical mapping of YAC inserts and high-molecular-weight genomic DNA. Lectures by outside speakers on topics of current interest supplemented the laboratory work.

PARTICIPANTS

Altenbach, S., Ph.D., USDA, Albany, California
Barbet, A., Ph.D., University of Florida, Gainesville
Campbell, M., B.S., Los Alamos National Laboratory, New Mexico
Dalrymple, M., Ph.D., Pharmaceutical Proteins Ltd., Edinburgh, United Kingdom
Forus, A., M.S., Norwegian Radium Hospital, Oslo
Fults, D., M.D., University of Utah, Salt Lake City
Imai, K., Ph.D., Max Planck Institute, Freiburg, Germany
Kamakura, T., Ph.D., University of California, Davis
Lindemann, G., B.S., University of Kansas Medical Center, Kansas City
McMillan, T., Ph.D., Institute of Cancer Research, Surrey, United Kingdom
Menancio-Hautea, D., Ph.D., University of Minnesota, St. Paul
Metcheva, I., Ph.D., Virginia Commonwealth University, Richmond
Saito, H., B.S., University of Wisconsin, Madison
Schorgendorfer, K., Ph.D., Biochemie GmbH, Kufstein, Austria
Sidenberg, D., Ph.D., Clarke Institute of Psychiatry, Toronto, Canada
Weitzel, J., M.D., Tufts New England Medical Center, Boston, Massachusetts

SEMINARS

Gardiner, K., Eleanor Roosevelt Institute. Pulsed-field gel electrophoresis and mammalian genome organization.
Klapholz, S., Cell Genesys, Inc. YAC cloning and yeast biology.
Lai, E., University of North Carolina. Pulsed-field gel electrophoresis.
Shizuya, H., California Institute of Technology. The BAC system: Cloning of human DNA in large fragments in *E. coli*.
Silverman, G., Harvard Medical School. YAC Characterization.
Smoller, D., Washington University. Cloning and characterization of a *Drosophila* P1 library.
Sternberg, N., DuPont Merck Pharmaceutical Co. Cloning with the P1 vector system.
Szybalski, W., University of Wisconsin. Sequencing without conventional cloning.

Protein Purification and Characterization

April 2–April 16

INSTRUCTORS

Burgess, Richard, Ph.D., University of Wisconsin, Madison
Guidotti, Guido, Ph.D., Harvard University, Cambridge, Massachusetts
Kadonaga, James, Ph.D., University of California, San Diego
Marshak, Daniel, Ph.D., Cold Spring Harbor Laboratory, New York

ASSISTANTS

Bankston, Laurie, Harvard University, Cambridge, Massachusetts
Croston, Glenn, University of California, San Diego
Kerrigan, Leslie, University of California, San Diego
Knuth, Mark, Promega Corporation, Madison, Wisconsin

This course was intended for scientists who are not familiar with techniques of protein isolation and characterization. It was a rigorous program that included laboratory work all day and a lecture with a discussion session every evening. Each student became familiar with each of the major techniques in protein purification by actually performing four separate isolations including (1) a regulatory protein from muscle tissue; (2) a sequence-specific, DNA-binding protein, (3) a recombinant protein overexpressed in *E. coli;* and (4) a membrane-bound receptor. A variety of bulk fractionation, electrophoretic, and chromatographic techniques were employed including precipitation by salts, pH, and ionic polymers; ion exchange, gel filtration, hydrophobic interaction, and reverse-phase chromatography; lectin affinity, oligonucleotide affinity, and immunoaffinity chromatography; polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis, and electroblotting; and high-performance liquid chromatography. Methods of protein characterization were utilized including

immunological and biochemical assays, peptide mapping, amino acid analysis, protein sequencing, and mass spectrometry. Emphasis was placed on strategies of protein purification and characterization rather than on automated instrumental analysis. Guest lecturers discussed protein structure, modifications of proteins, methodologies for protein purification and characterization, chemical synthesis of peptides, and applications of protein biochemistry to cell and molecular biology.

PARTICIPANTS

Bardwell, J., Ph.D., Harvard Medical School, Boston, Massachusetts
Bentley, D., Ph.D., ICRF, London, United Kingdom
Borchert, T., Ph.D., EMBL, Heidelberg, Germany
Bracete, A., Ph.D., Medical College of Virginia, Richmond
Brun, Y., Ph.D., Stanford University Medical School, California
Dorsky, D., Ph.D., University of Connecticut, Farmington
Farber, J., M.D., Johns Hopkins University, Baltimore, Maryland
Klasse, P.J., M.D., Chester Beatty Laboratories, London, United Kingdom
Liebhaber, S., M.D., University of Pennsylvania, Philadelphia
McMahon, M., Ph.D., DNAX Research Institute, Palo Alto, California
Menard, R., Ph.D., Biotechnology Research Institute, Montreal, Canada
Roulston, A., B.S., Lady Davis Institute, Montreal, Canada
Royer-Pokora, B., Ph.D., University of Heidelberg, Germany
Saccomano, N., Ph.D., Pfizer Research Inc., Groton, Connecticut
Tsai, J.-Y., Ph.D., Princeton University, New Jersey
Vernick, K., Ph.D., Carnegie Institution of Washington, Baltimore, Maryland

SEMINARS

Aebersold, R., University of British Columbia. Protein microsequencing.
Burgess, R., University of Wisconsin, Madison. Protein purification: Basic methods.
Casey, P., Duke University. Alkylation of proteins.
Erickson, B., University of North Carolina. Peptide synthesis.
Garrels, J., Cold Spring Harbor Laboratory. Two-dimensional gel electrophoresis.
Gierasch, L., University of Texas Southwestern Medical Center. Physical methods in protein analysis.
Hart, G., Johns Hopkins University. Glycosylation of proteins.
Kornberg, A., Stanford University. Why purify enzymes?
Kuret, J., and Tonks, N., Cold Spring Harbor Laboratory. Phosphorylation of proteins.
Pace, C.N., Texas A&M University. Protein denaturation.
Paterson, Y., University of Pennsylvania. Designing synthetic antigens.
Rose, G., University of North Carolina. Fundamentals of protein structure.
Rothman, J., Memorial Sloan Kettering Cancer Center. Protein trafficking.

Neurobiology of Human Neurological Disease

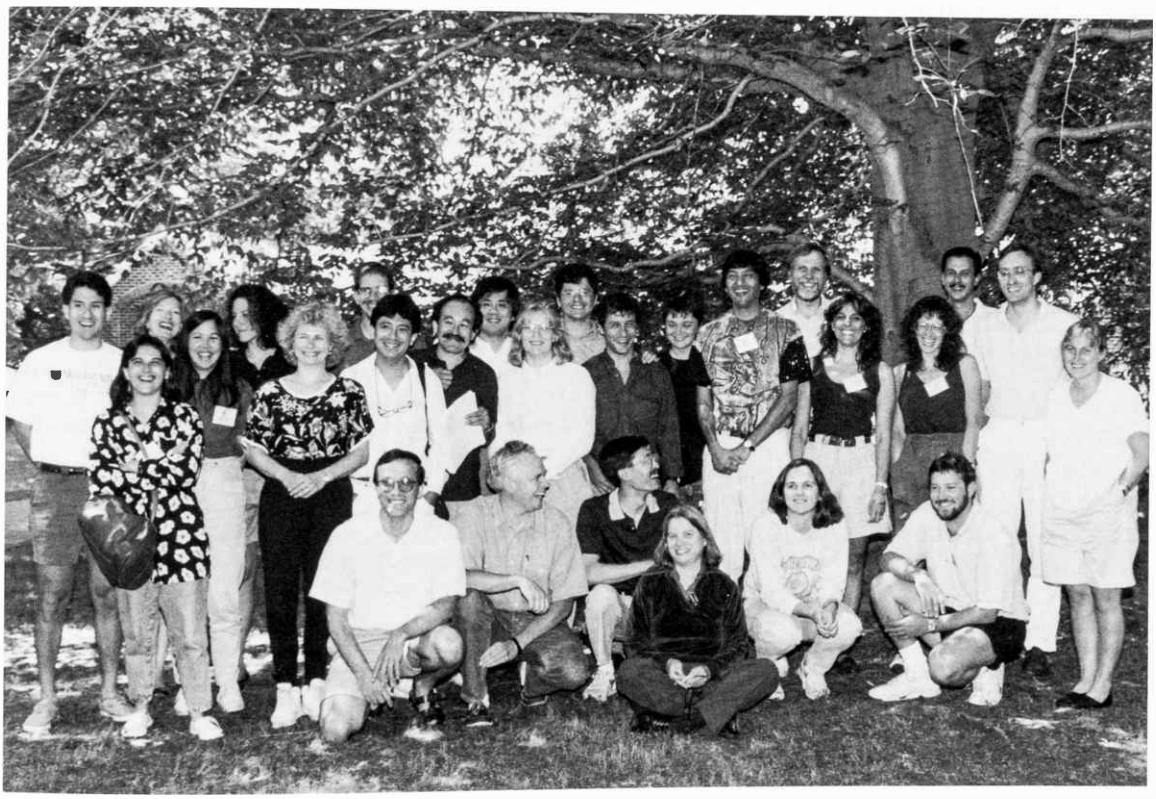
June 5–June 12

INSTRUCTORS

Choi, Dennis, Ph.D., Washington University School of Medicine
Mobley, William, Ph.D., University of California, San Francisco

This lecture course focused on selected arenas where recent advances in neuroscience have improved understanding of the pathogenesis of a human neurological disease. Substantial time was allotted to discussions led by the invited lecturers, so that in-depth delineation of specific approaches, methods, and results would be possible. An underlying general theme of the course was the exploration of the highly dynamic interface between basic neuroscience and clinical medicine. What can we learn about certain disease mechanisms by studying

molecules, cells, and animals, and what must we learn by studying patients? What experimental and clinical problems hinder the development of novel therapeutic approaches? Applicants with either neuroscience or clinical backgrounds were encouraged to apply.



PARTICIPANTS

Abosch, A., Ph.D., University of Pittsburgh, Pennsylvania
Behrens, M., M.D., Centro de Estudios Cientificos, Santiago, Chile
Carpi, D., B.S., Rutgers University, Piscataway, New Jersey
Delgado-Lezama, R., M.S., Cinvestav, Mexico City, Mexico
DePasquale, J., B.S., Rogoson Institute, New York, New York
Eide, F., M.D., University of California, San Francisco
Fields, K., Ph.D., National Institutes of Health, Rockville, Maryland
Flores, G., Ph.D., Cinvestav, Mexico City, Mexico
Garcia-Hernandez, F., Ph.D., Universidad Nacional Autonoma, Mexico City, Mexico
Gordon, J., B.A., University of California, San Francisco
Handran, S., B.S., Washington University, St. Louis, Missouri
Hyde, E., Ph.D., University of Pittsburgh, Pennsylvania
Jakeman, L., Ph.D., Syntex Research, Palo Alto, California
Lendon, C., Ph.D., St. Mary's Hospital, London, United Kingdom
Malaspina, D., M.D., New York State Psychiatric Institution, New York
Massieu, L., M.S., Universidad Nacional Autonoma, Mexico City, Mexico
Nunez, R., M.D., University of Pittsburgh, Pennsylvania
Oviedo, A., B.A., National Institutes of Health, Bethesda, Maryland
Sawa, A., M.D., University of Tokyo, Japan
Shafqat, S., B.S., Duke University, Durham, North Carolina
Swirnoff, A., B.S., Washington University, St. Louis, Missouri
Waksman, G., Ph.D., CNRS, Gif-sur-Yvette, France
Waldmann, C., B.S., Aidekman Research Center, Newark, New Jersey

SEMINARS

Barchi, R., University of Pennsylvania. Disorders of excitable membranes.

Chalfie, M., Columbia University. *C. elegans* neurodegenesis.

Choi, D., Washington University School of Medicine. Excitotoxicity and neurodegeneration.

Ciaranello, R., Stanford University. Brain development and psychiatric disorders.

Ferrendelli, J., Washington University School of Medicine. Principles of neuropharmacology and development of novel anticonvulsants.

Fields, H., University of California, San Francisco. New insights into mechanisms and treatment of pain.

Fischbeck, K., University of Pennsylvania. Molecular genetics of neuromuscular diseases.

Johnson, R., Johns Hopkins Hospital. Neurovirology and HIV encephal.

Landis, D., Case Western Reserve University. Role of glia in brain injury.

Mazziotta, J., University of California, Los Angeles. PET neuroimaging, functional anatomy, and neurological disease.

Mobley, W., University of California, San Francisco. Neurotrophic factors.

Monyer, H., University of Heidelberg. Glutamate receptors in health and disease.

Prusiner, S., University of California, San Francisco. Prion diseases of the nervous system.

Selkoe, D., Brigham & Women's Hospital. Alzheimer's disease.

Advanced Bacterial Genetics

June 5–June 25

INSTRUCTORS

Maloy, Stanley, Ph.D., University of Illinois, Urbana-Champaign
Stewart, Valley, Ph.D., Cornell University, Ithaca, New York
Taylor, Ronald, Ph.D., University of Tennessee, Memphis

ASSISTANTS

Allen, Scott, University of Illinois, Urbana-Champaign
Goldman, Barry, Cornell University, Ithaca, New York
Kaufman, Melissa, University of Tennessee, Memphis

This laboratory course demonstrated genetic approaches that can be used to analyze biological processes and their regulation, as well as detailed structure/function relationships of genes. Techniques covered included isolation, characterization, and mapping of mutations; use of transposable genetic elements as mutagens, linked selectable markers, and portable regions of homology; construction and analysis of operon and gene fusions; use of bacteriophage in genetic analysis; molecular cloning and restriction endonuclease mapping; allele exchange; Southern blot analysis; polymerase chain reaction; and site-specific mutagenesis. The course consisted of a series of experiments that employed these techniques in the genetic analysis of diverse bacterial and bacteriophage species. Lectures and discussions concentrated on the application of genetic analysis to contemporary questions in bacterial physiology, diversity, and pathogenesis.

PARTICIPANTS

Cottagnoud, P., Ph.D., Rockefeller University, New York, New York
Crockford, A., B.S., Imperial College of Science, London, United Kingdom
Flores, A., B.S., University of Seville, Spain
Frazee, R., B.S., University of Minnesota, Minneapolis
Grandjean, V., M.S., Institut de Genetique et Microbiologie, Orsay, France
Honeyman, A., Ph.D., Washington University, St. Louis, Missouri
Hsing, W., B.S., University of Massachusetts, Amherst
Kezdy, K., B.A., University of Alabama, Birmingham
Laalami, S., Ph.D., Institut de Biologie Physico-Chimique, Paris, France
Liao, J., Ph.D., Texas A&M University, College Station
Sanna, M., Ph.D., Sezione Scienze Microbiologiche, Rome, Italy
Sogaard-Andersen, L., Ph.D., University of Odense, Denmark
Tarkowski, T., B.A., University of Oregon, Eugene
Velterop, J., B.S., University of Amsterdam, The Netherlands
Villasenor, A., M.D. Hospital Infantil de Mexico, Mexico City
Waldor, M., Ph.D., Massachusetts General Hospital, Boston

SEMINARS

Gross, C., University of Wisconsin. DnaK and σ-^{70}: Global regulatory strategies in *E. coli*.
Hughes, K., University of Washington. 1. Using challenge phage to dissect the protein-DNA interactions that catalyze flagellar phase variation in *S. typhimurium*. 2. Genetic analysis of the regulatory cascade that controls flagellum biosynthesis in *S. typhimurium*.
Maloy, S., University of Illinois. The order of magnitude rule vs. physiological significance: Genetic analysis of *nac* in *K. aerogenes*.
Maurer, R., Case Western Reserve University. 1. Problems in bacterial DNA replication approached through genetics (and biochemistry). 2. What are we doing in here? A foray into *Salmonella* pathogenesis.
Ohman, D., University of Tennessee. 1. Genetic analysis in *P. aeruginosa:* Activation of genes for capsular polysaccharide production. 2. Genetics of funny bugs.
Salyers, A., University of Illinois. 1. Conjugal chromosomal elements in *Bacteriodes:* Sex without air. 2. Genetics of truly hilarious bugs.
Stewart, V., Cornell University. Genetic analysis of transmembrane signal transduction in *E. coli*.
Taylor, R., University of Tennessee. Genetics of *V. cholerae* virulence.

Molecular Approaches to Ion Channel Expression and Function

June 5–June 25

INSTRUCTORS

Enyeart, Jack, Ph.D., Ohio State University, Columbus
Goldin, Al, M.D., Ph.D., University of California, Irvine
Papazian, Diane, Ph.D., University of California, Los Angeles
Ruben, Peter, Ph.D., University of Hawaii, Honolulu

ASSISTANT

Fleig, Andrea, University of Hawaii, Honolulu

Application of the techniques of molecular biology to neurobiology has provided novel approaches and a new level of sophistication to the examination of many neurobiological problems. This intensive laboratory/lecture course was designed to introduce students to the application of model systems in which to express cloned neurotransmitter receptors and voltage-gated ion channels. The course covered the following topics: preparation of RNA transcripts in vitro and microinjection in *Xenopus* oocytes; in vitro mutagenesis of cloned ion channel genes; characterization of wild-type and mutated channels and receptors expressed in oocytes, using both two-microelectrode voltage-clamping and patch-clamping single-channel analysis; transient expression of voltage-gated ion channels in insect cells using baculovirus vectors; characterization of exogenous ion channels in insect cells using whole-cell patch clamping; and theory and analysis of ion currents.

PARTICIPANTS

Aggarwal, S., B.S., Harvard Medical School, Boston, Massachusetts
Bouvier, M., M.S., University College London, United Kingdom
Dourado, M., M.S., Florida State University, Tallahassee
Ffrench-Constant, R., Ph.D., University of Wisconsin, Madison
Hastings, G., Ph.D., National Institute on Alcohol Abuse, Rockville, Maryland
Price, C., B.S., University of Alberta, Canada
Ptacek, L., M.D., University of Utah, Salt Lake City
Saugstad, J., Ph.D., Oregon Health Science University, Portland
Willoughby, J., M.S., Sandoz Institute of Medical Research, London, United Kingdom
Xie, J., M.S., Rush Medical College, Chicago, Illinois

Advanced *Drosophila* Genetics

June 15–June 29

INSTRUCTORS

Ashburner, Michael, Ph.D., University of Cambridge, United Kingdom
Rubin, Gerald, Ph.D., University of California, Berkeley

This intensive seminar course provided an introduction to the theory and practice of methods used to manipulate the *Drosophila* genome. It was suitable for graduate students and researchers with some experience with *Drosophila* who are interested in expanding their knowledge of the wide range of genetic techniques now available for use with this organism. Topics covered included chromosome mechanics, the design and execution of genetic screens, and the use of transposable elements as genetic tools.

PARTICIPANTS

Anand, A., M.S., Indian Institute of Science, Bangalore
Asling, B., B.S., University of Stockholm, Sweden
Bermann, A., B.S., Max Planck Institute, Tubingen, Germany
Borisy, F., B.S., Johns Hopkins University, Baltimore, Maryland
Chang, H., B.S., University of California, Berkeley
Crittenden, J., B.S., Cold Spring Harbor Laboratory, New York
Dixon, L., Ph.D., Penn State University, University Park
Epstein, H., Ph.D., University of California, Davis
Kramer, H., Ph.D., University of California, Los Angeles
Lane, M., M.S., Columbia University, New York, New York
Martin, K., Ph.D., University of California, Los Angeles
Parker, L., Ph.D., Worcester Foundation, Shrewsbury, Massachusetts
Payre, F., Ph.D., CNRS, Toulouse, France
Prado, A., B.S., Instituto Cajal, Madrid, Spain
Risinger, C., B.S., Uppsala University, Sweden
Rutherford, S., B.A., University of California, San Diego
Schulz, C., B.S., Zoologisches Institut, Munich, Germany
Schweizer, R., M.S., Weizmann Institute, Rehovot, Israel
Serrano-Martinez, N., M.S., Institute Jacques Monod, Paris, France
Strathmann, M., Ph.D., Lawrence Berkeley Laboratory, California
Wassarman, D., Ph.D., Yale University, New Haven, Connecticut
Welbergen, P., Ph.D., York University, Ontario, Canada
Zamore, P., Ph.D., Harvard University, Cambridge, Massachusetts

ASSISTANTS

Conlon, Frank, Columbia University, New York, New York
Nichols, Jenny, University of Edinburgh, Scotland

This course was designed for molecular biologists, biochemists, and cell biologists interested in applying their expertise to the study of mouse embryonic development. Lecture and laboratory components of the course emphasized both the practical aspects of working with mouse embryos and the conceptual basis of current embryonic research. The following procedures and their potential applications were described: isolation and culture of germ cells and preimplantation and postimplantation embryos, embryo transfer, establishment, and genetic manipulation of embryo-derived stem cell lines, germ layer separation, chimera formation, nuclear transplantation, microinjection of DNA into eggs, retroviral infection of embryos, microinjection of cell lineage tracers, in situ hybridization, and immunohistochemistry. Guest lecturers discussed current research in the field.

PARTICIPANTS

Alessandrini, A., Ph.D., Harvard University, Cambridge, Massachusetts
Bermingham, J., Ph.D., University of California, San Diego
Bettler, B., Ph.D., The Salk Institute, San Diego, California
Bierkamp, C., M.S., University of Koln, Germany
Dymecki, S., Ph.D., Johns Hopkins University, Baltimore, Maryland
Federoff, H., Ph.D., Albert Einstein College of Medicine, New York, New York
Liao, X., Ph.D., Brandeis University, Waltham, Massachusetts
Lu, Y., Ph.D., Princeton University, New Jersey
Ma, A., M.D., Children's Hospital, Boston, Massachusetts
Reichardt, L., Ph.D., University of California, San Francisco
Shea, M., Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey
Spicer, D., Ph.D., Harvard Medical School, Boston, Massachusetts
Uchida, N., Ph.D., Stanford University, California
Wassarman, K., Ph.D., Yale University, New Haven, Connecticut

SEMINARS

Baltimore, D., Rockefeller University. Transcription: NF-κ genes and development.
Beddington, R., Centre for Genome Research. Postimplantation development.
Bradley, A., Baylor College of Medicine. Homologous recombination.
Bronner-Fraser, M., University of California, Irvine. Cell lineages in the neural crest.
Fraser, S., California Institute of Technology. Imaging cell lineages.
Hastie, N., MRC Human Genetics Unit. Human developmental mutations and mouse model systems.
Hogan, B., Vanderbilt University. Extraembryonic membranes.
Jackson, I., MRC Human Genetics Unit. Molecular genetics of mouse coat color: Black, white, brown, and blue mice.
Jessell, T., Columbia University. Establishing symmetry and pattern in the vertebrate nervous system.
Lovell-Badge, R., MRC National Institute for Medical Research. Sex determination II.
Mann, J., Beckman Research Institute of the City of Hope. Genetic imprinting in androgenetic and parthenogenetic ES cells.
McLaren, A., University College London. Germ cells and sex determination I.
McMahon, A., Roche Institute of Molecular Biology. *Wnt* genes.
Papaioannou, G., Tufts University. Chimeras/c-Fos.
Parada, L., NCI-FCRDC. Receptor tyrosine kinases: Important regulators of vertebrate development.
Rastan, S., MRC Clinical Research Centre. X-inactivation.
Rinchik, G., Oak Ridge National Laboratory. Genetic resources.
Robertson, L., Columbia University. ES cells: Biology and uses.
Rossant, J., Mount Sinai Hospital, Toronto. Preimplantation development.
Solter, D., Max Planck Institute, Freiburg. Genomic imprinting and totipotency.
Soriano, P., Baylor College of Medicine. Cellular oncogenes in development, etc.
Stewart, C., Roche Institute of Molecular Biology. The meaning of LIF.
Strickland, S., SUNY at Stony Brook. Oocyte maturation.
Waelsch, S., Albert Einstein College of Medicine. History of mouse genetics.
Wassarman, P., Roche Institute of Molecular Biology. Mammalian fertilization.
Wilkinson, D., National Institute for Medical Research. Segmentation in the vertebrate embryo.

SEMINARS

Bezanilla, P., University of California, Los Angeles. Voltage-dependent gating in ionic channels.

Enyeart, J., Ohio State University. 1. Peptide modulation of a novel K^+ channel in steroid-hormone-secreting cells. 2. Methods of voltage-clamp data analysis.

Goldin, A., University of California, Irvine. 1. Molecular aspects of sodium channel inactivation. 2. Electronics of the voltage and patch clamp. 3. Alternative expression systems.

Johnson, J., University of Pittsburgh. Modulation of the NMDA receptor by glycine and by intracellualr magnesium.

MacKinnon, R., Harvard Medical School. Structure/function studies in voltage-activated potassium channels.

Margiotta, J., Mt. Sinai Medical Center. Regulation of neuronal acetylcholine receptor function and distribution.

Papazian, D., University of California, Los Angeles. 1. Molecular biology techniques. 2. Structure and function of *shaker* potassium channels. 3. Baculovirus as an expression system.

Ruben, P., University of Hawaii. 1. Electrophysiology techniques. 2. Structure and function of sodium channels.

Sigworth, F., Yale University. How we spent two years studying one mutation of the potassium channel.

Trimmer, J., SUNY at Stony Brook. Molecular and cellular mechanisms regulating potassium channels.

White, M., University of Pennsylvania. Insights on the acetylcholine receptor.

Molecular Embryology of the Mouse

June 5–June 25

INSTRUCTORS

Lovell-Badge, Robin, Ph.D., National Institute for Medical Research, London, England
Parada, Luis F., Ph.D., Basic Research Program, NCI-FCRDC, Frederick, Maryland

CO-INSTRUCTORS

Mann, Jeff, Beckman Research Institute, Duarte, California
Rastan, Sohaila, Clinical Research Centre, Middlesex, England
Soriano, Phil, Baylor College of Medicine, Houston, Texas

SEMINARS

Ashburner, M., Cambridge University. Basic genetics; specialized chromosomes; genetic mapping methods.

Cline, T., University of California, Berkeley. Genetic screens as exemplified by screens for loci affecting sex determination: Genes and pathways.

Engels, W., University of Wisconsin. Genetics of PM hybrid dysgenesis and its control: Genetic behavior of P elements.

Gelbart, W., Harvard University. 1. Hobo mobile element. 2. *Drosophila* databases.

Glover, D., Dundee University. Genetic control of cell cycle; mitosis.

Hall, J., Brandeis University. Genetics of *Drosophila* behavior.

Hawley, S., University of California, Davis. Distributive segregation.

Lehmann, R., Whitehead Institute. Mutagenesis and genetic screens for maternal effect mutations.

Rubin, G., University of California, Berkeley. Somatic mosaics: Generation by X-irradiation and FLP/FRT; cell markers; data interpretation.

Spradling, A., Carnegie Institution. Use of P elements; heterochromatin.

Molecular Cloning of Neural Genes

June 29–July 19

INSTRUCTORS

Boulter, James, Ph.D., Salk Institute, San Diego, California
Chao, Moses, Ph.D., Cornell University, New York, New York
Eberwine, James, Ph.D., University of Pennsylvania, Philadelphia
Nef, Patrick, Ph.D., Salk Institute, San Diego, California

CO-INSTRUCTOR

Inman, Irene, Stanford University, California

ASSISTANTS

Kong, Hae-Young, University of Pennsylvania, Philadelphia
Lai, Cary, Salk Institute, San Diego, California

This intensive laboratory/lecture course was intended to provide scientists with a basic knowledge of the use of the techniques of molecular biology in examining questions of neurobiological significance. Particular emphasis was placed on using and discussing methods for circumventing the special problems posed in the study of the nervous system, for example, examination of low abundance mRNAs in extremely heterogeneous cell populations. The laboratory work included mRNA quantitation methods (nuclease protection, etc.), preparation of hybridization probes, library construction (λ ZAP and IST procedure), plaque screening techniques (probe hybridization, antibody interaction), DNA sequencing, PCR amplification, RNA amplification, and DNA-mediated gene transfer. A portion of the laboratory work was devoted to characterization and analysis of nucleic acids from single live neurons and glia. A major portion of the course was devoted to in situ hybridization and in situ transcription technologies. The lecture series, presented by invited speakers, focused on emerging techniques and how they may be applied to the study of the nervous system.

PARTICIPANTS

Ariano, M., Ph.D., Chicago Medical School, Illinois
Baptista, C., Ph.D., Columbia University, New York, New York
Cameron-Curry, P., Ph.D., Institut d'Embryologie Cellulaire, Nogent-sur-Marne, France
Dodson, B., M.D., University of California, San Francisco
Duerr, J., Ph.D., Oklahoma Medical Research Foundation, Oklahoma City
Fishell, G., Ph.D., Columbia University, New York, New York
Graybiel, A., Ph.D., Massachusetts Institute of Technology, Cambridge
Ingraham, C., Ph.D., Albany Medical College, New York
Lerea, L., Ph.D., Duke University, Durham, North Carolina
Malenka, R., Ph.D., University of California, San Francisco
Martone, M., Ph.D., University of California, San Diego
Parfitt, K., Ph.D., Stanford University Medical School, California
Rakhilin, S., Ph.D., University of Chicago, Illinois
Sheen, V., M.S., Children's Hospital, Boston, Massachusetts
Sipe, K., B.A., University of Illinois, Urbana
Wang, Y., Ph.D., Salk Institute, San Diego, California
Wexler, N., Ph.D., Columbia University, New York, New York
Wolszon, L., Ph.D., Columbia University, New York, New York

SEMINARS

Ballivet, M., University of Geneva. Nicotinic acetylcholine receptor gene expression.
Blakely, R., Emory University. Expression cloning of neurotransmitter transporters.
Cepko, C., Harvard Medical School. Cell-type development in the CNS.
Hatten, M., Columbia University. Control of granule cell neurogenesis and migration in developing cerebellar cortex.
Heinemann, S., Salk Institute. Glutamate receptors.
Lemke, G., Salk Institute. Molecular genetics of myelination.
Margolis, F., Roche Institute of Molecular Biology. Regulation of olfactory neural gene expression.
Morgan, J., Roche Institute of Molecular Biology. c-*fos* gene expression in the CNS.

Molecular and Developmental Biology of Plants

June 29–July 19

INSTRUCTORS

Cashmore, Anthony, Ph.D., University of Pennsylvania, Philadelphia
Klessig, Daniel, Ph.D., Rutgers University, Piscataway, New Jersey
Maliga, Pal, Ph.D., Rutgers University, Piscataway, New Jersey

ASSISTANTS

Tonkyn, John, Rutgers University, Piscataway, New Jersey
Bell, Callum, University of Pennsylvania, Philadelphia

Dixon, David, USDA, Southern Regional Research Center, New Orleans, Louisiana
Hennig, Jacek, Rutgers University, Piscataway, New Jersey
Klimczak, Les, University of Pennsylvania, Philadelphia
Matallana, Emilia, University of Pennsylvania, Philadelphia
Schindler, Uli, University of Pennsylvania, Philadelphia
Staub, Jeffrey, Rutgers University, Piscataway, New Jersey
Svab, Zora, Rutgers University, Piscataway, New Jersey

This course provided an intensive overview of current topics and techniques in plant biology, with an emphasis on molecular and developmental biology and genetics. It was designed for scientists with experience in molecular techniques who are working, or wish to work, with plant systems. The course consisted of a rigorous lecture series, a hands-on laboratory, and informal discussions. Guest speakers provided both an in-depth discussion of their work and an overview of their specialty. The laboratory sessions covered plant development; study of plasmodesmatal transport by microinjection; transient gene expression assays in protoplasts; transformation by *Agrobacterium* and the particle gun; in vitro reconstitution of protein transport into chloroplasts; in situ detection of RNA and protein; isolation of transcription factors and their characterization by DNA footprinting and methylation interference; modification of protein/DNA interaction by protein phosphorylation; pulsed-field gel electrophoresis and yeast artificial chromosomes in the analysis of the *Arabidopsis* genome.

PARTICIPANTS

Ainscough, J., B.S., Cambridge University, United Kingdom
Ait-Ali, T., M.S., Universite D'Orleans, France
Bellinncampi, D., Ph.D., Universita di Roma "La Sapienza," Italy
Chachulska, A., M.S., Institute of Biochemistry, Warsaw, Poland
Cruz-Mireles, R., M.S., Universidad Nacional, Cuernavaca, Mexico
Grossniklaus, U., M.S., University of Basel, Switzerland
Hodgins, R., Ph.D., University of Alberta, Canada
Iseli, B., M.S., Michigan State University, East Lansing
Lund, G., B.S., Istituto Biosintesi Vegetali, Milan, Italy
Machuka, J., M.S., University of Sussex, United Kingdom
Mollers, C., Ph.D., University of Gottingen, Germany
Quaedvlieg, N., B.S., University of Utrecht, The Netherlands
Sharma, S., Ph.D., DSIR-Grasslands, Palmerston, New Zealand
Wang, M., Ph.D., Leiden University, The Netherlands
Yi, Y., B.S., Dartmouth College, Hanover, New Hampshire

SEMINARS

Beachy, R., Scripps Research Institute. Plant RNA viruses: Molecular biology and applications.
Bisseling, T., Agricultural University, Wageningen, Netherlands. *Rhizobium*-plant symbiotic interactions.
Boynton, J., Duke University. The genetics and molecular biology of chloroplasts in *Chlamydomonas*.
Cashmore, A., University of Pennsylvania. Photoregulated gene expression.
Dooner, H., DNA Plant Technology Corporation. Transposable elements in plants.
Ecker, J., University of Pennsylvania. Yeast artificial chromosomes: A tool for the analysis of hormone pathways in *A. thaliana*.
Jones, A., University of North Carolina. Signal transduction in plants.
Jorgensen, R., University of California, Davis. Altered gene expression in plants due to trans-interactions between homologous genes.
Klessig, D., Rutgers University. Pathogen-plant interactions: Pathogenesis-related proteins.
Lamppa, G., University of Chicago. Protein targeting in plants.
Levings, S., North Carolina State University. The molecular biology of plant mitochondria: Cytoplasmic male sterility.
Lucas, W., University of California, Davis. Cellular communication by plasmodesmatal transport.
Maliga, P., Rutgers University. Transgenic plants.
McCarty, D., University of Florida. Seed development: Role of Vpl in regulation of seed maturation.
McDaniel, C., Rensselaer Polytechnic Institute. Developmental patterns in plants.
Nasrallah, J., Cornell University. Self-incompatibility in plants.
Nester, E., University of Washington. *Agrobacterium* biology and applications.
Quail, P., Plant Gene Expression Center. Phytochrome.
Schroeder, J., University of California, San Diego. Patch-clamp techniques and the study of ion-channel regulation in higher plants.
Somerville, C., Michigan State University. Biochemical genetics in *Arabidopsis*.
Theologis, A., Plant Gene Expression Center. Antisense RNA technology: Manipulation of plant hormone levels.
Varner, J., Washington University. The plant cell wall.
Yanofsky, M., University of California, San Diego. Molecular genetics of flower development.

Neurobiology of *Drosophila*

June 29–July 19

INSTRUCTORS

Hartenstein, Volker, Ph.D., University of California, Los Angeles
Ready, Don, Ph.D., Purdue University, West Lafayette, Indiana

ASSISTANTS

Green, Patricia, University of California, Los Angeles
Wolff, Tanya, Purdue University, West Lafayette, Indiana

This laboratory/lecture course was a series of in-depth discussions with researchers active in genetic, physiological, cellular, molecular, and behavioral studies of *Drosophila* neurobiology. It was organized around a core of seminars, extensive informal discussion, and lab work. Lecturers often brought original preparations for viewing and discussion, and directed lab exercises and experiments in their areas of special interest. The course was intended for researchers at all levels who want to use *Drosophila* as an experimental system for studying neurobiology.The course covered basic electrophysiological techniques, as applied to mutants with altered ion channels or sensory physiology. In addition, the course familiarized students with various preparations, including the embryonic nervous system, imaginal discs, and adult nervous system.

Topics included *Shaker* and the biophysics and diversity of potassium channels, the genetics and molecular biology of excitability, the control of

neurogenesis and neuronal diversity, embryonic and postembryonic development of the CNS and PNS, axonal pathfinding, development of the nervous system, mesoderm and muscle development, eye and optic lobe development, olfaction, learning, and the neural control of flight.

PARTICIPANTS

Engel, J., B.S., University of Iowa, Iowa City
Ferveur, J.-F., Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey
Garrity, P., B.A., California Institute of Technology, Pasadena
Hall, S., B.S., Purdue University, West Lafayette, Indiana
Harshman, K., Ph.D., Sloan Kettering Institute, New York, New York
Landgraf, M., B.S., University College London, United Kingdom
Seow, K., B.S., National University, Singapore
Silber, J., B.A., Washington University, St. Louis, Missouri
Skeath, J., B.A., University of Wisconsin, Madison
Stewart, B., M.S., University of Toronto, Canada
Urban, J., Ph.D., University of Mainz, Germany
Vinos, J., Ph.D., Universidad Autonoma, Madrid, Spain

SEMINARS

Bieber, A., Purdue University. Axonal pathfinding in the embryonic CNS.
Campos-Ortega, J., Institut Entwicklungsphysiologie. Neurogenic mutants.
Davis, R., Cold Spring Harbor Laboratory. Molecular basis of behavior.
Dickinson, M., University of Chicago. Introduction to physiology, sensory mechanisms, and aerodynamics of flight.
Doe, C., University of Illinois. CNS II: Neuronal fate.
Ganetzky, B., University of Wisconsin. Genetic dissection of membrane excitability.
Hartenstein, V., University of California, Los Angeles. 1. Introduction to *Drosophila* embryogenesis. 2. Early neurogenesis.
Keshishian, H., Yale University. Neuromuscular development.
Kidokoro, Y., University of California, Lcs Angeles. Synaptic physiology and receptors.
Lehmann, R., Whitehead Institute. Early pattern formation in *Drosophila*.
Ready, D., Purdue University. The morphogenetic furrow.
Strausfeld, N., University of Arizona. Adult CNS structure.
Taghert, P., Washington University. Neuromodulators and development of the stomatogastric NS.
Tomlinson, A., Columbia University. Eye development in the third instar.
Tully, T., Cold Spring Harbor Laboratory. Learning/behavior.
Wolff, T., Purdue University. Pupal eye development.
Zagotta, W., Stanford University. Ion channels in *Drosophila*.
Zuker, C., University of California, San Diego. Signal transduction.

Computational Neuroscience: Vision

July 10–July 24

INSTRUCTORS

Adelson, Edward, Ph.D., Massachusetts Institute of Technology, Cambridge
Heeger, David, Ph.D., NASA - Ames Research Center, Moffett Field, California
Hildreth, Ellen, Ph.D., Wellesley College, Massachusetts
Movshon, J. Anthony, Ph.D., New York University, New York

ASSISTANTS

Chichilinsky, E.J., Stanford University, California
Simoncelli, Eero, Massachusetts Institute of Technology, Cambridge

Computational approaches to neuroscience have produced important advances in our understanding of neural processing. Prominent successes have come in areas where strong inputs from neurobiological, behavioral and computational approaches can interact. Through a combination of lectures and hands-on experience with a computer laboratory, this intensive course examined several areas, including feature extraction, motion analysis, binocular stereopsis, color vision, higher-level visual processing, visual neural networks, and oculomotor function. The theme was that an understanding of the computational problems, the constraints on solutions to these problems, and the range of possible solutions can help guide research in neuroscience. Students had experience in neurobiological or computational approaches to visual processing. A strong background in mathematics was beneficial.

PARTICIPANTS

Beall, A., B.A., University of California, Santa Barbara
Boynton, G., M.A., University of California, Santa Barbara
Brady, N., B.A., Cornell University, Ithaca, New York
Braje, W., B.A., University of Minnesota, Minneapolis
Bricolo, E., B.S., Massachusetts Institute of Technology, Cambridge
Carandini, M., M.S., New York University, New York
Darrell, T., M.S., Massachusetts Institute of Technology, Cambridge
Econopouly, J., B.S., New York University, New York
Elder, J., B.S., McGill University, Montreal, Canada
Engel, S., B.A., Stanford University, California
Li, A., B.S., University of Rochester, New York
Losada, A., Ph.D., McGill University, Montreal, Canada
Mamassian, P., M.S., University of Minnesota, Minneapolis
Pauly, R., B.S., Florida State University, Tallahassee
Pece, A., Ph.D., University of Cambridge, United Kingdom
Pope, A., M.S., University of British Columbia, Vancouver, Canada
Prut, Y., B.S., Hebrew University, Jerusalem, Israel
Salzman, D., B.A., Stanford University, California
Sheinberg, D., B.A., Brown University, Providence, Rhode Island
Sinha, P., M.S., Massachusetts Institute of Technology, Cambridge
Smallman, H., M.S., University of California, San Diego
Tjan, B., B.S., University of Minnesota, Minneapolis
Young, M., M.S., New York University, New York

SEMINARS

Burt, P., David Sarnoff Research Laboratories. Models of visual attention and pattern recognition.
DeSimone, R., National Institutes of Health. Functional role of cortical neurons in attention and memory task.
Lisberger, S., University of California, San Francisco. Neural control and adaptive plasticity of smooth eye movements.
Malik, J., University of California, Berkeley. Computational models of visual texture processing.
McKee, S., Smith-Kettlewell Institute of Visual Science. Psychophysics and neurophysiology of stereoscopic vision.
Nakayama, K., Harvard University. Psychological studies of visual attention and memory.
Newsome, W., Stanford University School of Medicine. Neuronal mechanisms of visual motion perception.
Sejnowski, T., Salk Institute. Models of visual tracking.
Sparks, D., University of Pennsylvania. Superior colliculus and saccadic eye movements.
Von der Heydt, R., University Hospital Zurich. Neural processing of visual pattern and texture.
Wandell, B., Stanford University. Color vision: Psychophysics, physiology, and models.

Advanced Molecular Cloning and Expression of Eukaryotic Genes

July 21–August 10

INSTRUCTORS

Freedman, Len, Ph.D., Sloan Kettering Institute, New York, New York
Learned, Marc, Ph.D., University of California, Davis
Robbins, Alan, Ph.D., Tularik, Inc., South San Francisco, California

ASSISTANTS

Alroy, Iris, Sloan Kettering Institute, New York, New York
Feeser, Wendi, Dupont Merck Pharmaceutical, Wilmington, Delaware

This course focused on how to manipulate cloned eukaryotic genes to probe questions on their structure, expression, and function. As a model system, they examined *cis*- and *trans*-acting components involved in the regulation of eukaryotic gene expression. Students learned the theoretical and practical aspects of constructing genomic and cDNA libraries. Expression libraries from various organisms were screened with recognition site probes for specific DNA-binding proteins. A variety of transfection techniques were used to introduce cloned DNA molecules that had been manipulated in vitro into eukaryotic cells in culture. The expression pattern of these transfected DNAs were analyzed by

nuclease protection and enzymatic assays. Mutants were generated by oligo-directed and random mutagenesis procedures and characterized by DNA sequencing. Techniques and theory for detecting and characterizing the interaction between regulatory DNA sequences and *trans*-acting protein factors were presented. Guest lecturers discussed present problems in eukaryotic molecular biology as well as technical approaches to their solutions. Experience with basic recombinant DNA techniques was a prerequisite for admission to the course.

PARTICIPANTS

Bargonetti, J., Ph.D., Columbia University, New York, New York
Birken, S., Ph.D., Columbia University, New York, New York
Connor, L., B.S., Baylor College of Medicine, Houston, Texas
Dietmeier, K., B.S., Institut für Physiologische Chemie, Munich, Germany
Garry, D., Ph.D., University of Minnesota, Minneapolis
Grapin, A., M.S., Institut d'Embryologie du CNRS, Nogent-sur-Marne, France
Gratzer, S., B.S., University of Graz, Austria
Lorens, J., M.S., University of Bergen, Norway
Malofeeva, L., M.S., Moscow Physical Technical Institute, Russia
Powell, A., M.S., Ludwig Institute, London, United Kingdom
Rainwater, R., M.S., University of Alaska, Anchorage
Ross, C., Ph.D., Johns Hopkins Medical School, Baltimore, Maryland
Shiels, C., B.S., St. Mary's Hospital, London, United Kingdom
Smit, A., Ph.D., Vrije Universiteit, Amsterdam, The Netherlands
Suprenant, K., Ph.D., University of Kansas, Lawrence
Zimmermann, R., Ph.D., Max Planck Institute, Bad Nauheim, Germany

SEMINARS

Chandler, V., University of Oregon. The B locus of maize: A tissue-specific transcriptional activator.
DeLange, T., Rockefeller University. Molecular interactions at mammalian teleomeres.
Fields, S., SUNY at Stony Brook. A genetic system to detect protein-protein interactions.
Herr, W., Cold Spring Harbor Laboratory. Promoter-selective activation by transcription factors that bind to the same sequence.
Kingston, R., Massachusetts General Hospital. Transcription factor interactions on nucleosomal templates.
Landy, A., Brown University. Protein-DNA and protein-protein interactions in the higher-order complexes of λ site-specific recombination.
Rio, D., University of California, Berkeley. Mechanism and regulation of *Drosophila P*-element transposition.
Stillman, B., Cold Spring Harbor Laboratory. DNA replication in human and yeast cells.
Yamamoto, K., University of California, San Francisco. Non-receptor determinants of transcriptional regulation by glucocorticoids.

Imaging Structure and Function in the Nervous System

July 21–August 10

INSTRUCTORS

Katz, Larry, Ph.D., Duke University, Durham, North Carolina
Lewis, Richard, Ph.D., Stanford University, California

ASSISTANTS

Dolmetsch, Richard, Stanford University, California
McFarlane, Matthew, Stanford University, California
Yuste, Rafael, Bell Laboratories, Murray Hill, New Jersey

Recent advances in optical and video microscopy, coupled with the development of powerful new fluorescent probes, present unique opportunities for visualizing the structure and function of individual neurons and neuronal assemblies. This intensive laboratory/lecture course provided participants with the theoretical and practical tools to utilize these emerging technologies. Course topics included principles of fluorescence and video microscopy; image processing techniques; theory and practice of confocal microscopy; and the use of caged compounds to study intracellular and synaptic signaling. Particular emphasis was placed on the use of calcium-sensitive probes (e.g., fura-2, fluo-3) to monitor cell function in slices and in single cells. Using state-of-the-art equipment, students explored a variety of preparations, including mammalian brain slices, acutely dissociated neurons, and cultured cells. Applicants had a strong background in the neurosciences or cell biology.

PARTICIPANTS

Blum, K., Ph.D., Massachusetts Institute of Technology, Cambridge
Herrmann, K., Ph.D., University of California, Berkeley
Lachica, E., Ph.D., University of Washington, Seattle
Luo, L., Ph.D., Brandeis University, Waltham, Massachusetts
Miller, K., Ph.D., California Institute of Technology, Pasadena
Molnar, Z., M.D., Oxford University, United Kingdom
Schweizer, M., B.S., Max Planck Institute, Tubingen, Germany
Tcheng, T., B.S., University of Illinois, Urbana
Trommald, M., M.D., University of Oslo, Norway
Von Krosigk, M., Ph.D., Yale University, New Haven, Connecticut
Wexler, E., B.A., Albert Einstein College of Medicine, New York, New York
Zipser, B., Ph.D., Michigan State University, East Lansing

SEMINARS

Augustine, G., Duke University. Calcium dynamics and neurotransmission.
Bonhoeffer, T., Max Planck Institute. Optical recording and electrophysiology in cultured brain slices.
Connor, J., Roche Institute of Molecular Biology. High spatiotemporal resolution imaging of calcium in brain slices.
Fraser, S., California Institute of Technology. MRI and frontiers in imaging.
Grinvald, A., Weizmann Institute of Science. Optical imaging of architecture and function in the living brain.
Inoue, T., Universal Imaging Corporation. Design and implementation of imaging systems.
Lichtman, J., Washington University. Confocal microscopy of intact systems.
Nerbonne, J., Washington University. Principles an applications of caged compounds.
Ryan, T., Stanford University. Laser scanning confocal microscopy: Factors affecting image quality.
Steinbach, P., ETM Systems. Video image processor hardware.
Tank, D., Bell Laboratories. Modeling calcium dynamics in presynaptic terminals: Theory and experiment.
Tsien, R., Stanford University. Calcium oscillations in sympathetic neurons.
Tsien, R. W., University of California, San Diego. Design and function of fluorescent indicator dyes for calcium, other ions, and second messengers.
Wick, R., Hamamatsu Photonic Systems. Low- and high-sensitivity video cameras.

Yeast Genetics

July 21–August 10

INSTRUCTORS

Kaiser, Chris, Ph.D., Massachusetts Institute of Technology, Cambridge
Michaelis, Susan, Ph.D., Johns Hopkins University, Baltimore, Maryland
Mitchell, Aaron, Ph.D., Columbia University, New York, New York

ASSISTANTS

Roberg, Kevin, Massachusetts Institute of Technology, Cambridge
Sears, Dody, Johns Hopkins University, Baltimore, Maryland
Sia, Rey, Columbia University, New York, New York

The major laboratory techniques used in the genetic analysis of yeast were studied, including the isolation and characterization of mutants, tetrad analysis, complementation, and mitotic recombination. Micromanipulation used in tetrad analysis was carried out by all students. Molecular genetic techniques, including yeast transformation, gene replacement, analysis of gene fusions, and electrophoretic separation of chromosomes, were applied to the analysis of yeast DNA. Indirect immunofluorescence experiments were done to identify the nucleus, microtubules, and other cellular components. Lectures on fundamental aspects of yeast genetics were presented along with seminars given by outside speakers on topics of current interest.

PARTICIPANTS

Antes, T., B.S., University of California, Davis
Bartholomew, B., Ph.D., Southern Illinois University, Carbondale
Csank, C., Ph.D., National Research Council, Montreal, Canada
Edwards, J., M.D., University of California, Torrance
Eshel, D., Ph.D., University of Hawaii, Honolulu
Lee, S., Ph.D., University of California, San Fancisco
Ludewig, G., Ph.D., University of Kentucky, Lexington
Mala, C., M.S., Max Planck Institute, Martinsried, Germany
Radding, J., Ph.D., Eli Lilly & Co., Indianapolis, Indiana
Raleigh, E., Ph.D., New England Bio-Labs, Beverly, Massachusetts
Schwarz, E., Ph.D., Institut für Physiologisch Chemie, Munich, Germany
Sherman, M., Ph.D., Harvard Medical School, Boston, Massachusetts
Shimizu, M., Ph.D., Tokyo College of Pharmacy, Japan
Siebel, C., B.A., Whitehead Institute, Cambridge, Massachusetts
Smith, M., B.A., Columbia University, New York, New York
Zacharewski, T., Ph.D., University of Western Ontario, London, Canada

SEMINARS

Adams, A., University of Arizona. Genetic analysis of the yeast actin cytoskeleton.
Boeke, J., Johns Hopkins University. Yeast transposons.
Botstein, D., Stanford University. Molecular anatomy of yeast.
Carlson, M., Columbia University. Transcriptional control of glucose-repressed genes.
Fink, G., Whitehead Institute. Dimorphism in yeast: A new developmental pathway.
Futcher, B., Cold Spring Harbor Laboratory. Control points in the yeast cell cycle.
Hieter, P., Johns Hopkins University. Centromere function and chromosome stability.
Hoyt, A., Johns Hopkins University. Mitosis in *S. cerevisiae*.
Kaiser, C., Massachusetts Institute of Technology. Protein secretion in yeast.
Michaelis, S., Johns Hopkins University. The novel **a**-factor processing and secretion pathway.
Mitchell, A., Columbia University. Control of meiotic gene expression.
Petes, T., University of North Carolina. Homologous and nonhomologous recombination in yeast.
Rose, M., Princeton University. Nuclear fusion in yeast.
Sherman, F., University of Rochester. Transformation with synthetic oligonucleotides: Mechanisms and applications.
Sprague, G., University of California, Berkeley. Signal transduction in the mating response pathway.
Wigler, M., Cold Spring Harbor Laboratory. Functions of RAS and adenyl-cyclase-associated proteins.
Winston, F., Harvard Medical School. Regulators of Ty element transcription.

Molecular Neurobiology: Brain Development and Function

July 28–August 10

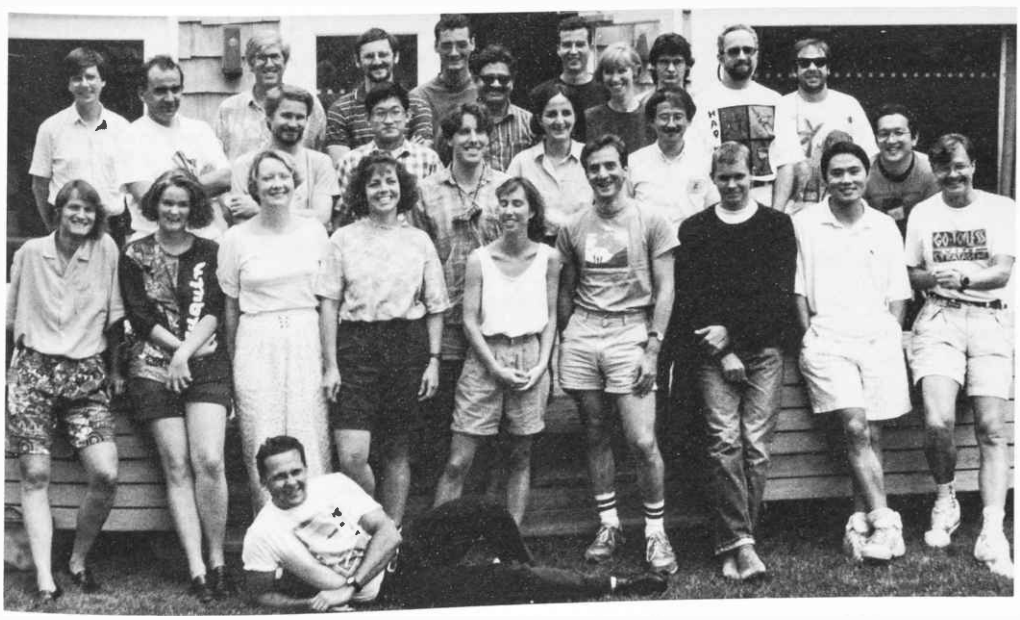
INSTRUCTORS

McKay, Ronald, Ph.D., Massachusetts Institute of Technology, Cambridge
Patrick, James, Ph.D., Baylor College of Medicine, Houston, Texas
Schwarz, Thomas, Ph.D., Stanford University, California

This lecture course presented both basic concepts and currently exciting research problems in molecular neurobiology. It focused on approaches and methods now used to study the development and function of the nervous system. Topics covered included gene expression, receptor structure and function, ion-channel cloning, second-messenger systems, learning, sensory transduction, behavioral genetics, neural induction, cell lineage, immortal cell lines, cell adhesion, oncogenes, and neurite outgrowth. The course provided the opportunity to discuss this rapidly expanding research area with invited lecturers. Individuals from a wide variety of backgrounds (graduate students to faculty) were encouraged to apply.

PARTICIPANTS

Agoston, D., M.D., National Institutes of Health, Bethesda, Maryland
Basta, D., Ph.D., Duke University, Durham, North Carolina
Bito, H., M.D., University of Tokyo, Japan
Cheyette, B., B.A., University of California, Los Angeles
Gale, H., M.S., Harvard University, Cambridge, Massachusetts
Gorman, M., B.A., Stanford University, California
Hay-Schmidt, A., Ph.D., University of Copenhagen, Denmark
Hoban, C., Ph.D., Massachusetts Institute of Technology, Cambridge
Jacobson, M., Ph.D., University College London
Jakowec, M., Ph.D., Yale University, New Haven, Connecticut
Jungbluth, S., B.S., Max Planck Institute, Martinsried, Germany
Kaiser, K., Ph.D., University of Glasgow, United Kingdom
Lardelli, M., Ph.D., Karolinska Institute, Stockholm, Sweden



LoPresti, P., M.D., SUNY at Stony Brook, New York
Maher, F., Ph.D., National Institutes of Health, Bethesda, Maryland
Maury, K., M.S., University of Geneva, Switzerland
Noda, M., Ph.D., Cancer Institute, Tokyo, Japan
Pan, D., B.S., University of California, Los Angeles
Schmucker, D., B.S., Max Planck Institute, Gottingen, Germany
Shifman, M., Ph.D., Mt. Sinai School of Medicine, New York, New York
Stapleton, G., B.S., Edinburgh University, United Kingdom
Van der Putten, H., Ph.D., Ciba-Geigy Research, Basel, Switzerland
Van Eekelen, J., Ph.D., University of Leiden, The Netherlands
Vandaele, S., Ph.D., University of Montreal, Canada
Yamada, G., Ph.D., Max Planck Institute, Gottingen, Germany

SEMINARS

Albright, T., Salk Institute. Functional diversity in the primate visual system: How we see motion, color, and form.
Bargmann, C., University of California, San Francisco. Genetic basis of chemosensation in *C. elegans*.
Byrne, J., University of Texas, Houston. Analysis of simple neural networks in *Aplysia*.
Campbell, K., University of Iowa. Dystrophin and its associated proteins.
Cline, H., University of Iowa. Activity-dependent events in the development of the topographic retinotectal projection.
Crews, S., University of California, Los Angeles. *Drosophila* CNS development.
Harris, K., Childrens Hospital, Boston. Synaptic integration.
Hatten, M., Columbia University. Cerebellar development.
Jahn, R., Yale University. Neurotransmitter release mechanism.
Jay, D., Harvard University. Laser inactivation of proteins during neural development.
Joyner, A., Mt. Sinai Hospital. Genetic approaches to neural development.
Julius, D., University of California, San Francisco. Serotonin.
Katz, L., Duke University. Development of neuronal connections in cortex.
McConnell, S., Stanford University. Determination of neuronal identities in the developing cerebral cortex.
McKay, R., Massachusetts Institute of Technology. Transplanting neuronal cells into the mammalian CNS.
Patrick, J., Baylor College of Medicine. Ligand gated ion channels.
Reed, R., Johns Hopkins University. Olfactory receptors.
Rutishauser, U., Case Western Reserve University. Regulation of cell interactions during neural development.
Schuman, E., Stanford University. Nitric oxide, intercellular signaling, and long-term potentiation.
Schwarz, T., Stanford University. Voltage-gated ion channels.
Simon, M., University of California, Berkeley. Signaling neuronal fate in the *Drosophila* eye.
Yancopoulos, G., Regeneron Pharmaceuticals. Neurotrophic factors.

Analysis and Genetic Manipulation of Yeast Artificial Chromosomes

October 8–October 21

INSTRUCTORS

Carle, Georges, Ph.D., Universite de Nice, France
Green, Eric, M.D., Ph.D., Washington University, St. Louis, Missouri
Rothstein, Rodney, Ph.D., Columbia University, New York, New York

ASSISTANTS

Canard, Bruno, Universite de Nice, France
Sunjevaric, Ivana, Columbia University, New York, New York
Tidwell, Rose, Washington University, St. Louis, Missouri

Cloning in yeast artificial chromosomes (YACs) is rapidly being applied to a wide variety of molecular genetic problems. This course provided basic scientific expertise in current techniques for the analysis and manipulation of YACs. In general, a blend of theoretical and practical information was provided, with the goal to establish a strong foundation for applying YAC cloning to a diversity of scientific

problems. Topics included standard yeast genetic techniques (such as the propagation and storage of cells, tetrad dissection, colony hybridization, and DNA transformation); YAC library screening using both PCR- and genetic-based strategies; characterizing YAC inserts by pulsed-field gel electrophoresis and PCR; manipulating YAC clones by recombination-mediated disruption, targeted integration, and YAC-YAC recombination; and techniques for introducing YACs into mammalian cells. Participants learned through hands-on experience, informal discussions, and lectures given by prominent experts designed to complement the experimental activities.

PARTICIPANTS

Avraham, K., Ph.D., National Cancer Institute, Frederick, Maryland
Borbye, L., M.S., Risoe National Lab, Roskilde, Denmark
Cook, G., Ph.D., Medical Research Council, Cambridge, England
Derry, J., Ph.D., Stanford University, California
Eyers, M., Ph.D., ICI Seeds, Berkshire, England
Ghazal, P., Ph.D., Scripps Research Institute, La Jolla, California
Hayashi, Y., Ph.D., ICRF, London, England
Heikoop, J., Ph.D., Leiden University, The Netherlands
Ji, H., Ph.D., University of Wisconsin, Madison
Myklebost, O., Ph.D., Institute for Cancer Research, Oslo, Norway
Rosier, M-F., B.S., CNRS, Villejuif, France
Shepel, L., Ph.D., University of Wisconsin, Madison
Sornson, M., B.A., University of California, San Diego
Thiesen, H.-J., Ph.D., Basel Institute for Immunology, Switzerland
Thomas, W., Ph.D., Genentech, Inc., South San Francisco, California
Wright, T., B.S., St. Mary's Hospital, London, England

SEMINARS

Bentley, D., Guy's Hospital, London. Isolation and utilization of YACs: Mapping human chromosomes X and 22.
Burke, D., University of Michigan. Development and uses of a mouse genomic YAC library.
Friedman, J., Rockefeller University. Genetic and physical mapping of mouse obesity genes.
Hieter, P., Johns Hopkins University. Modification of YACs using homologous recombination-based techniques.
Silverman, G., Children's Hospital, Boston. Expression of genes within YACs after their genetic transfer to mammalian hosts.
Vollrath, D., Whitehead Institute. Chromosome cartography: A high-resolution physical map of the human Y chromosome.

Macromolecular Crystallography

October 9–October 22

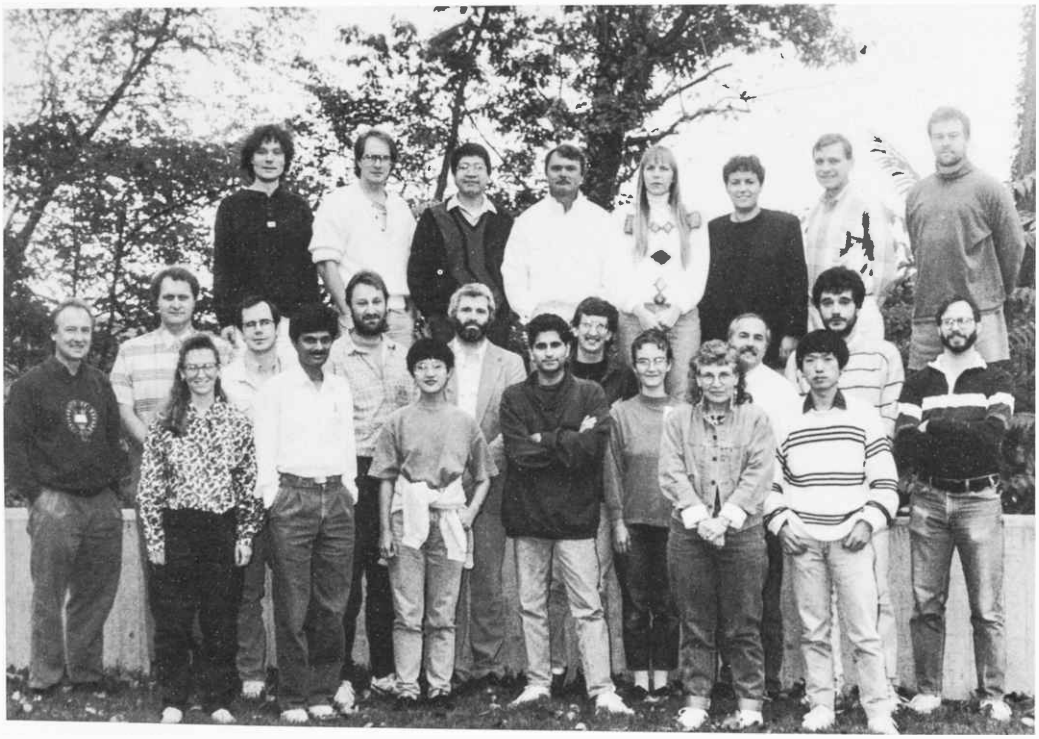
INSTRUCTORS

Furey, William, Ph.D., V.A. Medical Center, Pittsburgh, Pennsylvania
Gilliland, Gary, Ph.D., Center for Advanced Research in Biotechnology, Rockville, Maryland
McPherson, Alexander, Ph.D., University of California, Riverside
Pflugrath, James, Ph.D., Cold Spring Harbor Laboratory, New York

ASSISTANT

Dyda, Frederick, V.A. Medical Center, Pittsburgh, Pennsylvania

Crystallography and X-ray diffraction yield a wealth of structural information unobtainable through other methods. This intensive laboratory/computational course focused on the major techniques used to determine the three-dimensional structures of macromolecules. It was designed for scientists with a working knowledge of protein structure and function, but who are new to macromolecular crystallography. Topics covered included protein purification, crystallization, crystal characterization, data collection, data reduction, anomalous dispersion, phase determination, molecular replacement and averaging, electron density interpretation, structure refinement, molecular graphics, and molecular dynamics. Participants learned through extensive hands-on experiments, informal discussions, and lectures on current applications of these and related procedures given by outside speakers.



PARTICIPANTS

Almog, O., M.S., Hebrew University, Jerusalem, Israel
Darst, S., Ph.D., Stanford University, California
Edwards, A., Ph.D., McMaster University, Hamilton, Canada
Gajiwala, K., M.S., Texas A&M University, College Station
Hassell, A., M.S., Glaxo Research Institute, Research Triangle Park, North Carolina
Heikinheimo, P., M.S., University of Turku, Finland
Huber, A., B.S., California Institute of Technology, Pasadena
Jin, L., B.S., Boston University, Massachusetts
Li, T., Ph.D., Johns Hopkins University, Baltimore, Maryland
Olkowski, J., B.S., Eli Lilly & Company, Indianapolis, Indiana
Roca, A., B.S., University of Wisconsin, Madison
Thomas, L., M.S., SUNY at Buffalo, New York
Vanderhoff-Hanaver, P., Ph.D., University of Tennessee, Oak Ridge
Weisgraber, K., Ph.D., Gladstone Institute, San Francisco, California
Wilce, M., Ph.D., St. Vincent's Institute, Melbourne, Australia
Zhang, W., M.S., Albert Einstein College of Medicine, New York, New York

SEMINARS

Brunger, A., Yale University. The accuracy of X-ray crystal and solution NMR structures.
Clore, G. M., National Institutes of Health. Determination of high-resolution structures of larger proteins by 3D and 4D NMR.
Fitzgerald, P., Merck Sharp & Dohme Research Laboratories. HIV-protease: A target for structure-based drug design.
Furey, W., V.A. Medical Center. Structure determination of pyruvate decarboxylase.
Gilliland, G., Center for Advanced Research in Biotechnology. 1. The crystal structure of a gluthathione *S*-ransferase from a Mu gene class. 2. Crystal structure determinations and analysis of subtilisin BPN mutants for studying thermal stability.
Holden, H., University of Wisconsin, Madison. The three-dimensional structure of myosin subfragment-1.
Kjeldgaard, M., Aarhus University. The structure of elongation factor Tu-GDP.
Kuriyan, J., Rockefeller University. A sliding clamp and a clamping switch: X-ray structures of DNA polymerase processing factor and the SH2 domain.
McPherson, A., University of California, Riverside. The structure of satellite tobacco mosaic virus.
Otwinowski, Z., Yale University. Data reduction and profile fitting.
Ramakrishnan, V., Brookhaven National Laboratory. The MAD method to determine protein structure: Application to histone H5.
Sweet, R., Brookhaven National Laboratory. Laue diffraction as a probe for in-crystal dynamics: The example of guanido-benzoyl trypsin.
Tronrud, D., University of Oregon. Macromolecular refinement.
Wilson, I., Scripps Research Institute. Immune recognition of peptide antigens.

Advanced In Situ Hybridization and Immunocytochemistry

October 9–October 22

INSTRUCTORS

Hough, Paul, Ph.D., Brookhaven National Laboratory, Upton, New York
Jacobson, Ken, Ph.D., University of North Carolina, Chapel Hill
Spector, David, Ph.D., Cold Spring Harbor Laboratory, New York
Trask, Barbara, Ph.D., University of Washington School of Medicine, Seattle

ASSISTANTS

Derby, Robert, Cold Spring Harbor Laboratory, New York
Lipfert, Jennifer, University of North Carolina, Chapel Hill

This course focused on specialized techniques and concepts in microscopy related to localizing nucleic acid sequences and proteins in cells and preparing isolated nucleic acids for microscopic examination. The course was designed for the molecular biologist who is in need of microscopic cell biological approaches and for the cell biologist who is not familiar with the practical application of the

advanced techniques presented in the course. The course emphasized the use of the latest techniques and methods in epifluorescence microscopy, confocal laser scanning microscopy, and electron microscopy to localize nucleic acids and proteins in mammalian cells. Among the methods presented were preparation of tagged nucleic acid probes, fixation methods, detection of multiple nucleic acids in a single cell, chromosome spreads, use of a variety of reporter molecules and nonantibody fluorescent tags, direct and indirect antibody labeling, and detection of multiple proteins in a single cell. In addition, electron microscopic techniques to observe isolated nucleic acids and proteins were presented. For each technique, several experimental protocols were presented allowing the students to assess the relative merits of each and to relate them to their own research. The students were encouraged to bring specific probes to the course which could be used in addition to those provided by the instructors. The laboratory portion of the course was supplemented by invited lecturers who gave up-to-the-minute reports on current research using the techniques being presented in the course.

PARTICIPANTS

Bridge, J., M.D., University of Nebraska, Omaha
Chen, C., M.S., Columbia University, New York, New York
Chu, M.-L., Ph.D., Thomas Jefferson University, Philadelphia, Pennsylvania
Eccles, M., Ph.D., University of Otago, Dunedin, New Zealand
Fan, F., B.S., University of Southern California, Los Angeles
Kaech, S., Ph.D., National Institutes of Health, Bethesda, Maryland
Lakoski, J., Ph.D., University of Texas, Galveston
McDonough, K., Ph.D., Albert Einstein College of Medicine, New York, New York
McKinnon, D., Ph.D., SUNY at Stony Brook, New York
Pauletti, G., Ph.D., University of California, Los Angeles
Rosen, B., Ph.D., University of Edinburgh, United Kingdom
Shinowara, N., Ph.D., Winthrop University Hospital, Mineola, New York
Solc, C., Ph.D., Massachusetts General Hospital, Boston
Tuor, U., Ph.D., Hospital for Sick Children, Toronto, Canada
White, M., Ph.D., University of Oxford, United Kingdom

SEMINARS

Brinkley, W., Baylor College of Medicine. Ultrastructural organization of the kinetochore.

Hansma, P., University of California, Santa Barbara. Imaging of biological samples with the atomic force microscope.

Hough, P., Brookhaven National Laboratory. 1. Image production in the electron miocroscope. 2. Analysis of macromolecular complexes by STEM.

Jacobson, K., University of North Carolina. 1. Basic introduction to light microscopy. 2. Fluorescence microscopy.

Murray, J., University of Pennsylvania. Use of confocal microscopy and deconvolution techniques.

Singer, R., University of Massachusetts. Cytoplasmic organization of mRNA.

Spector, D., Cold Spring Harbor Laboratory. 1. Immunocytochemistry. 2. An integrated microscopic approach to examining nuclear organization.

Trask, B., University of Washington School of Medicine. Mapping chromosomes and interphase nuclei by in situ hybridization.

Waggoner, A., Carnegie Mellon University. Development of fluorochromes and filters for fluorescence microscopy.

Ward, D., Yale University. 3D cytogenetics and cell biology.

Essential Computational Genomics for Biologists

October 26–November 4

INSTRUCTORS

Branscomb, Elbert, Ph.D., Lawrence Livermore Laboratory, California
Goodman, Nat, Ph.D., Massachusetts Institute of Technology
Marr, Tom, Ph.D., Cold Spring Harbor Laboratory, New York
Myers, Gene, Ph.D., University of Arizona

This course was intended primarily for molecular biologists and geneticists who are mounting large-scale projects that require informatics components. No prior experience with the use of computers or mathematics was assumed. Lectures and computer work delved deeply into both the theoretical issues and practical approaches to a number of important computational problems in genomic analy-

sis. The course included an overview of informatics systems analysis and design principles. Topics included such basics as database design and data modeling to more complex analytical methods. Technical issues relating to the analysis of raw data such as image analysis, sequence assembly, physical mapping, genetic-linkage analysis, and data integration were covered. Students were introduced to, and gained hands-on experience with, a variety of software tools used in computer systems design and data acquisition and analysis running on Macintosh computers. It was intended that students gain sufficient knowledge and experience from this course to enable them to initiate active collaborations with computational scientists at their home institutions.

PARTICIPANTS

Caporale, L., Ph.D., Merck & Co., Inc., Rahway, New Jersey
Darvasi, A., M.S., Hebrew University, Jerusalem, Israel
Davies, P., Institut Pasteur, Paris, France
Glamann, J., M.D., State Seruminstitute, Copenhagen, Denmark
Gnirke, A., Ph.D., University of Washington, Seattle
Kanyion, P., Ph.D., Justus-Liebig University, Giessen, Germany
Lagunez-Otero, J., Ph.D., Weizmann Institute, Rehovot, Israel
Meisler, M., Ph.D., University of Michigan, Ann Arbor
Reiner, O., Ph.D., Baylor College of Medicine, Houston, Texas
Ringwald, M., Ph.D., Max Planck Institute, Freiburg, Germany
Sabour, M., Ph.D., Agriculture Canada, Ottawa
Schmitt, K., M.S., University of Southern California, Los Angeles
Williams, C., Ph.D., Weyerhaeuser Research, New Bern, North Carolina

Molecular Genetics, Cell Biology, and Cell Cycle of Fission Yeast

October 27–November 9

INSTRUCTORS

Chappell, Tom, Ph.D., Duke University, Durham, North Carolina
Fantes, Peter, Ph.D., University of Edinburgh, Scotland
McLeod, Maureen, Ph.D., State University of New York, Brooklyn

ASSISTANTS

Ayscough, Kathryn, ICRF, London, England
Devoti, James, State University of New York, Brooklyn
Wu, Shaw-Yun, State University of New York, Brooklyn

Recent advances in cell cycle research, largely due to studies on the fission yeast *Schizosaccharomyces pombe*, have prompted much interest in this organism. Although cell cycle studies continue to flourish, *S. pombe* is increasingly being chosen as a model organism for investigations into other aspects of cell biology and genetics using the powerful molecular and genetical techniques available. The content of the course reflected all these areas of interest and provided participants with the skills necessary to pursue their own investigations. Topics covered included mutagenesis and analysis of mutants, transformation and gene transplacement techniques, isolation of nuclei, preparation of nuclear DNA, plasmid recovery from yeast into bacteria, cell cycle methods,

cytology, and immunological techniques. In addition to hands-on experience, participants had the opportunity to learn through informal group discussions and formal lectures given by prominent *S. pombe* researchers drawn from the expanding international community.

PARTICIPANTS

Albright, C., Ph.D., Whitehead Institute, Cambridge, Massachusetts
Bachhawat, A., Ph.D., Carnegie Mellon University, Pittsburgh, Pennsylvania
Belenguer, P., Ph.D., University Paul Sabatier, Toulouse, France
Brun, C., Ph.D., Roswell Park Cancer Institute, Buffalo, New York
Dhillon, N., Ph.D., Salk Institute, La Jolla, California
Gulli, M-P., Ph.D., Lab de Biologie du CNRS, Toulouse, France
Hoffmann, L., M.S., University of Copenhagen, Denmark
Lum, P., M.S., University of Washington, Seattle
Miller, P., B.S., University of Vermont, Burlington
Moussy, G., M.S., CNRS, Villejuif, France
Rasmussen, C., Ph.D., University of Alberta, Canada
Romano, P., Ph.D., National Institutes of Health, Bethesda, Maryland
Saltsman, K., B.A., Massachusetts General Hospital, Boston
Smith, A., Ph.D., Whitehead Institute, Cambridge, Massachusetts
Smythe, C., Ph.D., University of Dundee, Scotland
Su, J.-Y., Ph.D., University of Colorado, Denver

SEMINARS

Caligiuri, M., Cold Spring Harbor Laboratory. The role of Cdc10 in the cell cycle.
Chappell, T., Duke University. Golgi-localizing proteins.
Davey, J., University of Birmingham. Sex appeal and endocytosis in *S. pombe*.
Fantes, P., University of Edinburgh. Cell cycle control in fission yeast.
Futcher, B., Cold Spring Harbor Laboratory. Cell cycle clocks in *S. cerevisiae*.
Gould, K., Vanderbilt University. Cell cycle regulation of the cdc2 protein kinase.
Hoffman, C., Boston College. Glucose repression and signal transduction in *S. pombe*.
Klar, A., NCI Frederick Cancer Facility. Decisions cells make during mating-type switching of fission yeast.
McLeod, M., SUNY at Brooklyn. Protein kinases that regulate meiosis.
Subramani, S., University of California, San Diego. Role of *S. pombe rad* genes in DNA repair, recombination and cell-cycle checkpoints.
Wigler, M., Cold Spring Harbor Laboratory. Studies of RAS in yeast.
Young, P., Queen's University. Na, pH, and the cell cycle.

Monoclonal Antibodies from Combinatorial Libraries

October 27–November 9

INSTRUCTORS
Barbas, Carlos, Ph.D., Scripps Research Institute
Burton, Dennis, Ph.D., Scripps Research Institute

ASSISTANTS
Cababa, Doug, Scripps Research Institute
Pilkington, Glenn, Scripps Research Institute

Recent advances in the generation and selection of antibodies from combinatorial libraries allow for the rapid production of antibodies from immune and nonimmune sources. This laboratory/lecture course focused on the construction of combinatorial antibody libraries expressed on the surface of phage and selection of desired antibodies from pre-existing libraries by panning. Students learned the theoretical and practical aspects of constructing combinatorial libraries from immune and nonimmune sources as well as the construction of synthetic antibody libraries. Production, purification, and characterization of Fab fragments expressed in *E. coli* were also covered. Students were encouraged to select antibodies against an antigen of their interest from the pre-existing libraries. The lecture series, presented by a number of invited speakers, focused on the biology of filamentous phage and the utility of surface expression libraries, expression of antibodies in *E. coli* and mammalian cells, antibody structure and function, antibody diversity, catalytic antibodies, and recent results on the use of antibodies in therapy.

PARTICIPANTS

Abate, M., M.S., Dartmouth Medical School, Lebanon, New Hampshire
Bjorling, E., B.S., Karolinska Institute, Stockholm, Sweden
Bodeus, M., Ph.D., INSERM, Paris, France
Deutscher, S., Ph.D., University of Missouri, Columbia
Godenir, N., M.S., University of Cape Town, South Africa
Greenspan, N., Ph.D., Case Western Reserve University, Cleveland, Ohio
Herlyn, D., D.V.M., The Wistar Institute, Philadelphia, Pennsylvania
Logan, J., Ph.D., EPG Laboratories, Manhasset, New York
Ou, S., M.S., California Institute of Technology, Pasadena
Shepard, A., Ph.D., Alkermes Inc., Boston, Massachusetts
Shiba, K., Ph.D., Cancer Institute, Tokyo, Japan
Takkinen, K., Ph.D., VTT Laboratory, Espoo, Finland
Titlow, C., Ph.D., Massachusetts General Hospital, Boston
Torrance, L., Ph.D., Scottish Crop Research Institute, Dundee
Tse, W., Ph.D., Stanford University, California
Williams, D., Ph.D., Kennedy Institute, London, United Kingdom

SEMINARS

Barbas, C., Scripps Research Institute. Antibodies from combinatorial libraries.
Burton, D., Scripps Research Institute. 1. Antibody structure and function. 2. Human antibodies from combinatorial libraries.
Dimmock, N., University of Warwick. Antibody neutralization of animal viruses.
Glennie, M., University of Southampton. Antibodies in human therapy.
Hilvert, D., Scripps Research Institute. Catalytic antibodies.
Matthews, D., Genentech, Inc. Optimization of binding and catalysis by phage display technology.
Model, P., Rockefeller University. Filamentous phages.
Sanz, I., University of Texas. Generation of antibody diversity.
Scott, J., University of Missouri. Discovering peptide ligands using epitope libraries.
Wilson, I., Scripps Research Institute. Antibody-antigen interaction at the molecular level.

Molecular Markers for Plant Breeding

November 11–November 22

INSTRUCTORS

Burr, Ben, Ph.D., Brookhaven National Laboratory, Upton, New York
Helentjaris, Tim, Ph.D., University of Arizona, Tucson
Tingey, Scott, Ph.D., DuPont Experimental Station, Wilmington, Delaware

ASSISTANTS

Jung, Mark, DuPont Experimental Station, Wilmington, Delaware
Hanafey, Mike, DuPont Experimental Station, Wilmington, Delaware
Matz, Eileen, Brookhaven National Laboratory, Upton, New York
McCreery, Tom, University of Arizona, Tucson

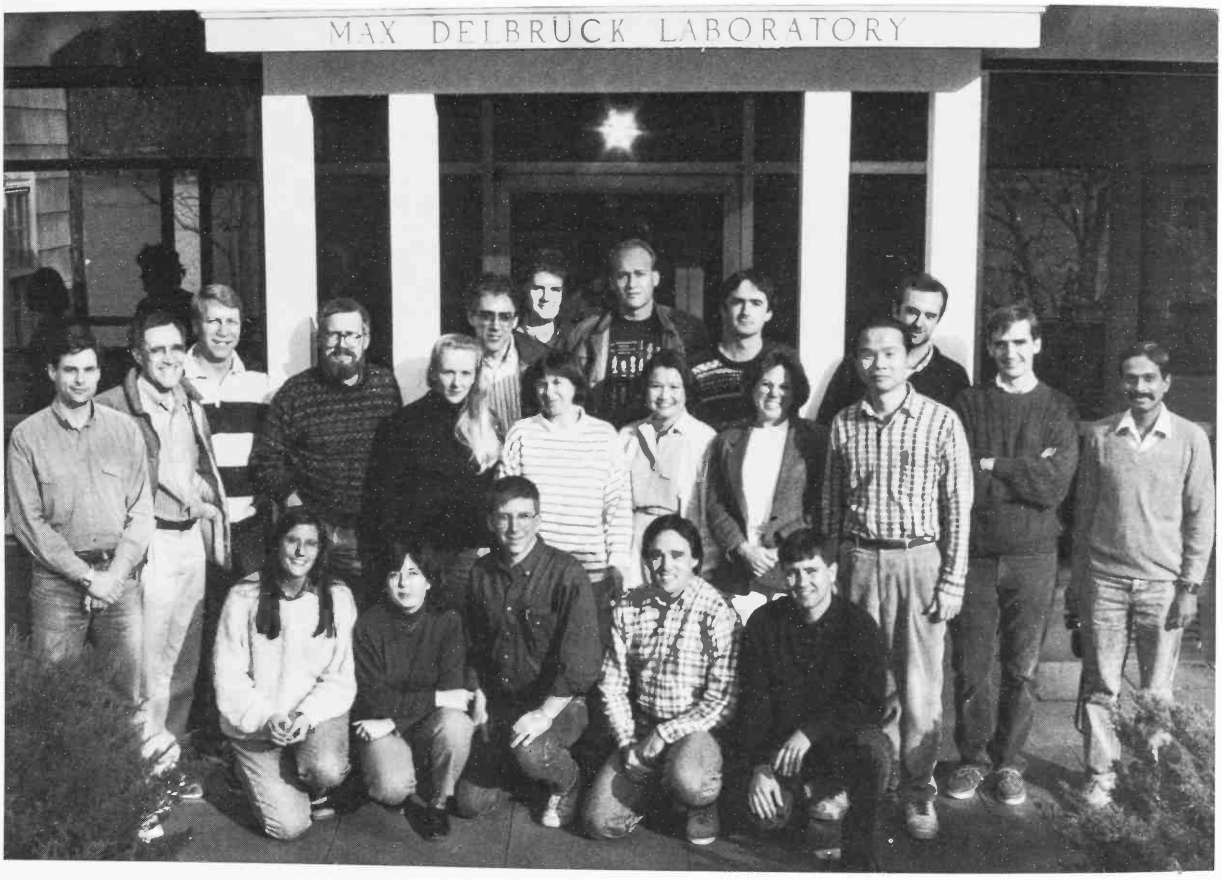
This course was designed to train scientists in the use of molecular markers in plant breeding. It was taught through both a theoretical and practical approach to the subject, including lectures and extensive laboratory work. Participants learned a variety of techniques that can be used to approach problems such as single gene introgression, analysis of genetic diversity, gene mapping, and quantitative trait analysis. These techniques included DNA preparation, non-radioisotopic Southern analysis, PCR, RAPDs, and DNA sequencing. The course included training in computational analysis of both RFLP and RAPD data and taught methods from a variety of plant and animal systems with an emphasis on potential future strategies. Course participants were expected to have knowledge of, but not necessarily practical experience with, the basic principles of molecular biology and genetics.

PARTICIPANTS

Andrade, E., B.S., Max Planck Institute, Koln, Germany
Chang, Y.-F., M.S., CIBA-GEIGY Corp., Research Triangle Park, North Carolina
Cheah, S.-C., Ph.D., Palm Oil Research Institute, Lumpar, Malaysia
Chomet, P., Ph.D., Dekalb Plant Genetics, Mystic, Connecticut
Doerge, R., M.S., North Carolina State University, Raleigh
Farish, G., M.S., University of North Dakota, Grand Forks
Grattapaglia, D., B.S., North Carolina State University, Raleigh
Hubbard, L., B.A., USDA-ARS, Albany, California
Messenguer, R., Ph.D., IRTA Cabrils, Barcelona, Spain
Morgante, M., Ph.D., University of Udine, Italy
Murigneux, A., M.S., BIOCEM, Aubierre, France
Nair, S., Ph.D., International Centre for Genetic Engineering, Delhi, India
Rameau, C., Ph.D., INRA, Versailles, France
Schneerman, M., M.S., Illinois State University, Bloomington
Van Deynze, A., M.S., University of Guelph, Canada

SEMINARS

Beckman, J., Centre d'Etude du Polymorphisme Humain, Paris. Molecular mapping in mammals.
Burr, B., Brookhaven National Laboratory. Use of molecular markers for plant genetics.
Ecker, J., University of Pennsylvania. Map based cloning; creating anchored physical maps.
Lander, E., Whitehead Institute. Use of molecular markers for genome analysis.
Michelmore, R., University of California, Davis. Future applications of molecular markers.
Rafalski, A., DuPont Experimental Station. 1. Polymorphism detection techniques. 2. Automation technology.
Romero-Severson, J., Agrigenetics. The measurement of linkage in heredity.
Sederoff, R., North Carolina State University. Application of molecular markers to perennials.
Smith, S., Pioneer Hi-Bred International. Use of molecular markers in germ plasm analysis.



SEMINARS

Cold Spring Harbor Seminars were initiated to provide a semiformal avenue for communication between the various research groups at the Laboratory. They are particularly useful for research personnel who have joined the Laboratory during the summer. The seminars also afford a necessary opportunity for the graduate students and postgraduate staff to develop their skills in defending, organizing, and presenting their research.

1992

January

Sergei Sokol, Harvard University, Cambridge Massachusetts. Growth factors and the body plant formation in *Xenopus* early development.

Karen Fien, Cold Spring Harbor Laboratory. Identification of RFC from *Saccharomyces cerevisiae*: A component of the leading-strand DNA replication complex.

Melanie Cobb, University of Texas, South Western Medical Center. Regulation of MAP kinases by insulin and EGF.

Grigori Enikolopov, Cold Spring Harbor Laboratory. Recombinant inhibitors of protein kinases: New tools for studying signal transduction.

Will Phares, Cold Spring Harbor Laboratory. HIV-1 LTR sequences amplified from peripheral blood cells of infected individuals contain a "hot spot" for sequence duplication.

February

Tom Steitz, Yale University, New Haven, Connecticut. Comparison of the structures of HIV reverse transcriptase Klenow fragment: Implications for function.

Elizabeth Moran, Cold Spring Harbor Laboratory. Adenovirus E1A oncogene intervention in cell growth control pathways.

Paul Schimmel, Massachusetts Institute of Technology, Cambridge. Enzymatic recognition of RNA minihelices and decoding genetic information.

Ian Fitch, Cold Spring Harbor Laboratory. Cloning and characterization of four B-type cyclin genes, CLB1–CLB4, from *Saccharomyces cerevisiae*.

Janet Rossant, Mt. Sinai. Anterior/posterior patterning in the postimplementation mouse embryo.

Jacek Skowronski, Cold Spring Harbor Laboratory. Altered T-cell activation and development in transgenic mice expressing the HIV-1 *nef* gene.

Elizabeth Ullu, Yale University, New Haven, Connecticut. RNA processing reactions in African trypanosomes.

Adrian Krainer, Cold Spring Harbor Laboratory. Regulation of pre-mRNA splice site selection by antagonistic factors.

March

Matt Thomas, Washington University, St. Louis, Missouri. Protein tyrosine phosphatases and T-cell activation.

Jim Pflugrath, Cold Spring Harbor Laboratory. T7 lysozyme: An amidohydrolase that inhibits T7 RNA polymerase.

Michael Sinensky, Eleanor Roosevelt Institute, Denver, Colorado. Prenylated proteins of the mammalian nuclear lamina.

Steve Bell, Cold Spring Harbor Laboratory. ATP-dependent recognition of *S. cerevisiae* origins of DNA replication.

Susan Amara, Vollum Institute. Molecular diversity of neurotransmitter transporters.

Mark Pittenger, Cold Spring Harbor Laboratory. Cellular and molecular aspects of tropomyosin diversity.

April

Jeff Hall, Brandeis University, Waltham, Massachusetts. How a gene keeps time: Molecular neurobiology of period in *Drosophila*.

Dorre Grueneberg, Cold Spring Harbor Laboratory. Interaction of a novel human homeodomain protein with SRF (serum response factor).

Dan Littman, Howard Hughes Medical Institute. CD4 and CD8 in T-cell development and activation.

George Mulligan, Cold Spring Harbor Laboratory. RNA-binding proteins in the regulation of β-tropomyosin alternative splicing.

Jack Dixon, University of Michigan, Ann Arbor. Structure, function, and catalysis of protein tyrosine phosphatases.

Sui Huang, Cold Spring Harbor Laboratory. Nuclear organization of pre-mRNA splicing factors and their substrates.

May

Susan Lobo, Cold Spring Harbor Laboratory. snRNA gene transcription by RNA polymerases II and III.

Lea Harrington, Cold Spring Harbor Laboratory. Purification and characterization of *Tetrahymena* telomerase.

Erich Grotewold, Cold Spring Harbor Laboratory. Transcription regulation by a maize Myb homolog: The *P* gene.

June

Ricardo Attar, Cold Spring Harbor Laboratory. Expression cloning of a novel zinc finger protein that binds to the c-fos serum response element.

October

Nikolai Lisitsyn, Cold Spring Harbor Laboratory. Genomic difference analysis: A representational approach.

James Darnell, Rockefeller University, New York, New York. IFN activation of genes: Signal transduction by protein-protein interaction.

Dan Kalderon, Columbia University, New York, New York. Genetic investigation of A-kinase function in *Drosophila*.

Yue Xiong, Cold Spring Harbor Laboratory. Human D-type cyclins associate with multiple protein kinases and PCNA.

David Levin, Johns Hopkins University, Baltimore, Maryland. Role of the protein kinase C/MAP kinase pathway in yeast.

Simon Green, Cold Spring Harbor Laboratory. A novel RNA-binding motif in the double-stranded RNA-activated protein kinase, DAI.

November

Jiminez Garcia, Cold Spring Harbor Laboratory. Spatial organization of transcription and pre-mRNA splicing in adenovirus-infected cells.

Jo Messing, Waksman Institute. Methionine overproduction and a new somatic mutation in maize.

Andrew Flint, Cold Spring Harbor Laboratory. The non-transmembrane protein tyrosine phosphatase PTB1B is a target for three different serine/threonine kinases in vivo.

December

Wen-Hwa Lee, University of Texas Health Science Center. What's new with RB?

Danny Reinberg, Robert Wood Johnson Medical School at Rutgers University, New Brunswick, New Jersey. Regulation of initiation of transcription by polymerase II.

Peter Yaciuk, Cold Spring Harbor Laboratory. Characterization of the E1A-associated 300-kilodalton host cell protein and its associated cell growth regulating activity.

Eckard Wimmer, State University of New York, Stony Brook. CAP-independent translation and cell-free, de novo synthesis of poliovirus.

UNDERGRADUATE RESEARCH

An important aspect of the summer program at the Laboratory is the participation of college undergraduate students in active research projects under the supervision of full-time laboratory staff members. The program was initiated in 1959. Since that year, 378 students have completed the course, and many have gone on to creative careers in biological science.

The objectives of the program are to provide (1) a greater understanding of the fundamental principles of biology; (2) an increased awareness of major problem areas under investigation; (3) a better understanding of the physical and intellectual tools for modern research and the pertinence of this information to future training; and (4) a personal acquaintance with research, research workers, and centers for study.

The following students, selected from more than 160 applicants, took part in the program, which was supported by Baring Brothers & Co., Ltd., Bio-Rad Laboratories, Burroughs Wellcome Fund, Theodore N. Danforth, The Garfield Internship, Hanson Industries, Imperial Chemical Industries, Libby Internship, National Science Foundation, Robert H.P. Olney Memorial Cancer Fund, Philips Petroleum Foundation., Inc., William Shakespeare Internship, and Frederica von Stade Internship.

Kenneth Bilchick, Dartmouth College
Advisor: **John Anderson**
Sponsor: National Science Foundation
Purification, crystallization, and structure determination of M*Pvu*II methylase protein.

Chad Brecher, Brown University
Advisor: **Dan Marshak**
Sponsor: Burroughs Wellcome Fund
Examination of the mechanism of action of S100β and determination of the interaction of S100β and β-amyloid (1–40) in the C6 rat glioma cell line and newborn rat astrocytes.

Daniel Cahill, Yale University
Advisor: **Jim Pflugrath**
Sponsor: Burroughs Wellcome Fund
Structure determination of S100β.

Howard Y. Chang, Harvard University
Advisor: **Gil Morris**
Sponsor: Robert H.P. Olney Memorial Cancer Fund
Mechanism of *trans*-activation of the human PCNA promoter by the 243-amino-acid E1A protein.

Victor Wee-Teck Chua, Cambridge University
Advisor: **Adrian Krainer**
Sponsor: Bio-Rad Laboratories
In vivo functional analysis of the general splicing factors SF2 and hnRNP A1.

Leena Gandhi, University of Utah
Advisor: **Arne Stenlund**
Sponsor: Burroughs Wellcome Fund
Analysis of the interaction between E1 and E2 in cooperative binding of the BPV origin of replication.

Keow Lin (Lindee) Goh, California Institute of Technology
Advisor: **Robert Martienssen**
Sponsor: Philips Petroleum Foundation, Inc.
Molecular cloning and characterization of the *ramosa1* mutant of maize.

Sam Haward, Cambridge University
Advisor: **Venkatesan Sundaresan**
Sponsor: Baring Brothers & Co., Ltd.
Devising and testing a screening system for selecting for transposable element insertions into *Arabidopsis* plants.

Gilbert L. Henry, University of Califorinia at Santa Barbara
Advisor: **Winship Herr**
Sponsor: National Science Foundation
Transcriptional activation domains.

JoAnn Hong, Yale University
Advisor: **Dafna Bar-Sagi**
Sponsor: Frederica von Stade Fund
The effect of GRB2 overexpression in NIH-3T3 fibroblast cells containing moderately high levels of wild-type p21 RAS.

Fraser R. Imrie, University of Glasgow
Advisor: **Tim Tully**
Sponsor: Hanson Industries
Molecular cloning of *linotte*, a new learning and memory gene in *Drosophila melanogaster*.

Ingrid Kelly, University of Cambridge
Advisor: **Tom Peterson/Erich Grotewold**
Sponsor: Anonymous
Purification of antibodies to the *P* gene in maize.

Laurie Littlepage, University of North Texas
Advisor: **Bruce Futcher**
Sponsor: National Science Foundation
Suppressing the lethality of WHI3 overexpression in *S. cerevisiae*.

Rachna J. Ram, University of California, Berkeley
Advisor: **Hong Ma**
Sponsor: Burroughs Wellcome Fund
Differential hybridization screening for floral organ-specific cDNAs in *Arabidopsis thaliana*.

Rustam Rea, Oxford University
Advisor: **Nick Tonks/Andrew Flint**
Sponsor: Libby Internship
Investigation of the phosphorylation of two cytosolic protein tyrosine phosphastases.

Adam Todd Ross, University of Michigan
Advisor: **B. Robert Franza**
Sponsor: The Garfield Internship
Initial characterization of human I-κB and other Rel-associated proteins.

Anjanette Searfoss, Juniata College
Advisor: **Kim Arndt**
Sponsor: National Science Foundation
Cloning of SAP-4, a SIT4-associated protein.

Anna Sessler, Allegheny College
Advisor: **Jeff Kuret**
Sponsor: National Science Foundation
Cellular localization of the protein kinase CKI-1 in *Saccharomyces cerevisiae*.

Wenying Shou, Pomona College
Advisor: **Tom Marr**
Sponsor: William Shakespeare Internship
Dispersed pattern recognition in a group of proteins.

Rebecca Smith, Bard College
Advisor: **Bruce Stillman**
Sponsor: National Science Foundation
Elucidation of ORC binding to *S. cerevisiae* ARS1 through high-resolution footprinting.

Michael J. Walsh, Tuskegee University
Advisor: **Eric Richards**
Sponsor: National Science Foundation
Study of variant telomere repeats in *Arabidopsis thaliana*.

Jennifer Whangbo, University of North Carolina, Chapel Hill
Advisor: **Ron Davis**
Sponsor: National Science Foundation
An approach to studying the molecular basis of behavior in mammals through the use of promoter trap mice.

Lucie L.-N. Yang, University of Maryland
Advisor: **David M. Helfman**
Sponsor: Cold Spring Harbor Laboratory
Identification of a cellular factor blocking splicing of a skeletal-muscle-specific exon in nonmuscle cells.

NATURE STUDY PROGRAM

The Nature Study Program gives elementary and secondary school students the opportunity to acquire a greater knowledge and understanding of their environment. Through a series of specialized field courses, younger students can engage in introductory programs such as Nature Bugs and Nature Detectives I and II, and older students can enroll in more advanced programs such as Marine Biology and Nature Photography.

During the summer of 1992, a total of 420 students participated in 29 courses within the program; 17 of these courses were filled to capacity. The classes were held outdoors, weather permitting, at the Uplands Farm Preserve, the headquarters of the Long Island Chapter of the Nature Conservancy. The Laboratory has equipped and maintains classroom and laboratory facilities as well as a darkroom at Uplands Farm. This facility is used as a base for the students' exploration of the local environment. Field classes are held on Laboratory grounds, St. John's Preserve, Fire Island National Seashore, Caumsett State Park, the Cold Spring Harbor Fish Hatchery and Aquarium, as well as other local preserves and sanctuaries.

In addition to the three 2-week sessions, the Adventure Education course meets on two Saturdays for trips. The students go on a 10-mile bicycle hike to Sagamore Hill and a 12-mile canoe trip on the Nissequogue River.

PROGRAM DIRECTOR

William M. Payoski, M.A., Adjunct Professor, Nassau Community College

REGISTRAR

Catherine Schratwieser, M.A., Dowling College

INSTRUCTORS

Kimberly Hamilton, B.S., Colgate University
Michael Manfredonia, Teaching Candidate, Marist College
Linda Payoski, B.A., Science Teacher, Uniondale High School
Marjorie Pizza, B.A., Science Teacher, Bayville School District
Donna Stokes, Teaching Candidate
Sarah Whiteside, Photography Undergraduate

COURSES

Nature Bugs
Nature Detectives I and II
Advanced Nature Study
Ecology Explorers
Frogs, Flippers, and Fins
Pebble Pups
Rock Hounds
Bird Study
Freshwater Life
Seashore Life
Marine Biology
Nature Photography
Adventure Education

The Laboratory would like to acknowledge the generosity of the following companies who loaned equipment and reagents to the various courses:

Amersham Corp.
Applied Biosystems, Inc.
AT-Biochem
Axon Instruments
Beckman Instruments, Inc., Fullerton Division
Beckman Instruments, Inc., Spinco Division
Bio 101, Inc.
Biometra
Bio-Rad Laboratories
Bio-Rad, Microscience Division
Boehringer Mannheim Biochemicals
Brinkmann Instruments, Inc.
CBS Scientific
Clontech Laboratories, Inc.
Dagan Corp.
Denton Vacuum
Diagnostic Products Corp.
Drummond Scientific Corp.
Eastman Kodak Co.
Electron Microscopy Sciences
Eppendorf Inc.
ETM Systems
FMC Corporation
Fuji Medical Systems
General Valve Corporation
GIBCO
Grass Instrument Co.
Hampton Research
Hoefer Scientific Instruments
International Biotechnologies, Inc. (IBI)
ICN Pharmaceuticals, Inc.
Integrated Separation Systems
IntelliGenetics, Inc.
Kramer Scientific
Micro Video Instruments
Millipore Corp.
Mitsubishi Electronics America, Inc.
MJ Research, Inc.
New England Biolabs, Inc.
Newport
Nikon Inc.
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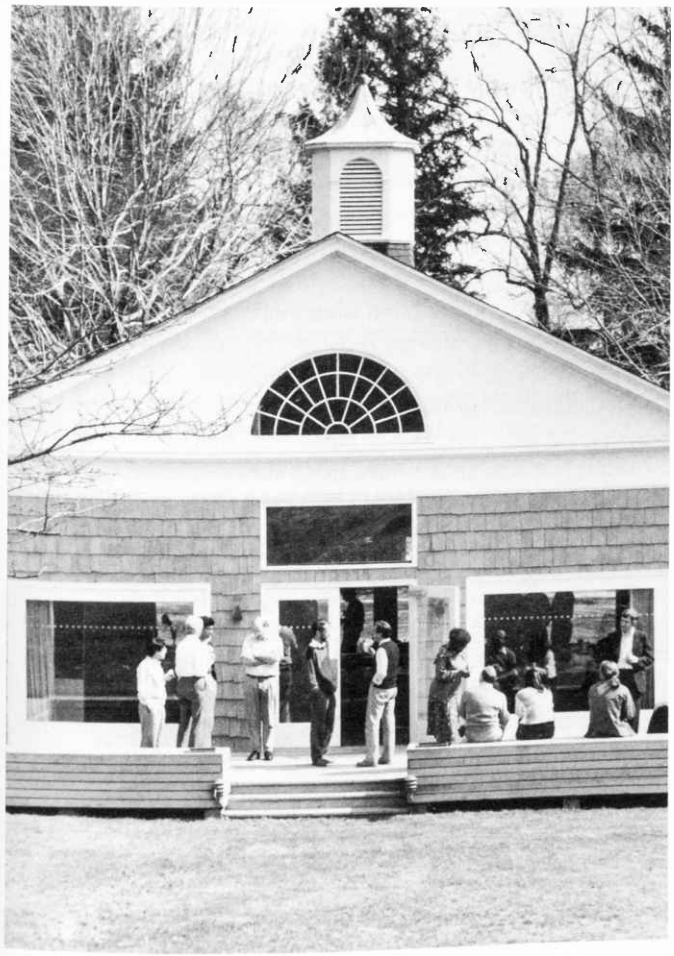
Omega Optical
Organon Technika-Cappel
Perkin Elmer Cetus
Polysciences
Promega Corp.
Q-Life Systems
Qiagen Inc.
Robbins Scientific Corp.
SAS Institute
Savant Instruments, Inc.
Schleicher & Schuell, Inc.
Stratagene
Sutter Instrument Co.
Tektronix
United States Biochemical Corp.
Vector Laboratories, Inc.
Warner
World Precision Instruments
Carl Zeiss, Inc.

BANBURY CENTER

BANBURY CENTER DIRECTOR'S REPORT

1992 marked the 15th anniversary of the opening of the Banbury Center, donated to Cold Spring Harbor Laboratory in 1975 by Mr. Charles S. Robertson. What had been the garage for the Robertson estate was converted into the conference room during 1976, and Francis Crick was the inaugural speaker at the dedication that took place on June 14, 1977. Jim Watson, in his annual report for that year, wrote of the new building that "we believe (it) to be the most striking conference center now available to biologists." His confidence has been fully justified by the enthusiasm of researchers for our meetings and the high esteem in which the Banbury program is held by biologists throughout the world. The reputation of the Center has spread beyond academia into the worlds of biotechnology and science policy, sometimes with unexpected consequences. Our 15th year was celebrated with 16 meetings attended by more than 500 visitors.



Banbury Conference Center

Robertson House provides housing and dining accommodations at Banbury Center

Sloan Foundation Workshops

A description of the year's events must begin with a special tribute to the Alfred P. Sloan Foundation for the support it has provided to the Banbury Center program. This support goes back to the very beginning of the program with a grant that, together with a similar grant from the Esther A. and Joseph Klingenstein Fund, provided the bedrock for the first two years of the Center's existence. Victor McElheny, the Center's first director, then persuaded the Sloan Foundation that science education of influential groups was essential for a civilized society and thus began the series of "public information workshops" for science journalists and congressional staff. These workshops provided an opportunity for the participants to spend 2 days with leading scientists and learn in depth about matters of biology that had important public policy implications. This series proved to be a tremendous success, and two of these workshops have been held every year since 1980. The workshops have been very influential because the participants are opinion leaders, ideally placed to transmit what they learned at Banbury.

The two workshops in 1992 exemplified the quality and the importance of these small meetings. **The Commercialization of Biology** meeting dealt with the vexing question of the degree to which research in molecular biology and genetics is being compromised by the rush to exploit the findings. Speakers came from industry, the National Institutes of Health, academia, and the Patents & Trademarks Office. It was at this meeting that the changes in licensing for the polymerase chain reaction were first made public. Later in the spring, there was a meeting on **Occupational and Environmental Health**. Ill health related to the workplace places enormous economic strain on the nation and personal cost on those individuals affected. Improvements in occupational health should be a major target for any administration concerned with reducing health care costs, and this meeting introduced the science journalists and congressional staff to leading workers in the field.

The Alfred P. Sloan Foundation decided, regretfully, that all good things must come to an end. The Foundation will not extend funding for the program beyond 1992, although there will be one more workshop in 1993. So at the same time that we thank the Foundation for its insight and forward thinking in supporting these workshops, we are actively looking for future long-term support for the program.

New Technical Developments in Molecular Biology

The first of the meetings funded by the Corporate Sponsors Program dealt with a topic of great general interest and of special interest to biotechnology and pharmaceutical companies, namely, producing protein molecules with desired characteristics. "Rational" design strategies have not been very effective because the huge number of possible variants of a protein precludes systematic testing of all forms, and our lack of knowledge of the relationships between protein function and structure hinder prediction of which variants to select for testing. The **Phage Display** meeting discussed a new strategy that involves cloning the gene for the protein to be modified into bacteriophage so that the protein is displayed on the surface of the virus. The gene is randomly mutated, and phage bearing a variant protein with characteristics closer to those desired are selected from the enormous population of phage that can be grown in bacteria. The first papers on this technique appeared during 1991, and this was almost certainly the first meeting to discuss these new developments. The meeting set a new Banbury Center record for biotechnology participation with no fewer than 31 of the 45 participants coming from companies!

Molecular Biology

The other meetings funded by the Corporate Sponsor program were all concerned with molecular biology and the ways it has illuminated our understanding of the biology of organisms.

An intriguing biological problem with important medical implications concerns the mechanisms by which some viruses, having infected cells, become dormant. How is it that the DNA of these viruses remains in the infected cells, although the cells continue to function normally? Equally intriguing and important are the mechanisms by which this DNA later becomes activated. **Molecular Mechanisms of Viral Latent Infections** reviewed these topics in relation to the herpesviruses, Epstein-Barr virus, and, of course, human immunodeficiency virus and other RNA viruses.

One way that viral infections might be controlled is by using oligonucleotides that hybridize to and shut down viral genes. This "antisense" strategy has been under intensive development for a number of years, but some new developments led us to hold the meeting **Oligonucleotide Manipulation of Gene Expression: Its Therapeutic Potential**. These developments include the synthesis of oligonucleotides that are more stable when they are in cells and that make more stable combinations with DNA and RNA; further information on the ways in which oligonucleotides enter cells that may in turn lead to improved methods of delivery; and new data on the formation of triple helices.

The meeting **Mechanisms of Neuronal Survival: The Action of Neurotrophic Factors** dealt with the fascinating topic of growth factors that affect nerve cell growth and survival. Sessions discussed not only the factors them-

selves, but also their receptors; regulation of their activities in vivo; their role in development; and the mechanisms by which nerve cells are killed. There is clearly great potential for developing therapies to induce or promote nerve regeneration following nerve damage.

The Wellcome Trust/Cold Spring Harbor Laboratory Meeting

The Wellcome Trust is the principal non-Government source of funding for biomedical research in the United Kingdom and has recently begun to hold meetings. **Constructing Organisms** was a joint meeting of The Wellcome Trust and the Laboratory, the Trust providing funds to cover the costs of the large contingent of European scientists who attended. The meeting brought together researchers working on a veritable zoo of creatures, including fruit flies, mice, zebra fish, nematode worms, sea urchins, toads, leeches, and weeds! Participants examined the extent to which Nature employs common developmental strategies in building organisms. We hope that this may be the first of other joint meetings.

Human Molecular Genetics

In something of a departure compared with recent years, there was but one meeting dealing directly with human genetics. However, the meeting, entitled **DNA Repeats and Human Gene Mutations**, was extremely timely, providing an opportunity to bring together scientists working on three different inherited disorders. The molecular defects in these disorders—Fragile X syndrome (the most common form of inherited mental retardation); myotonic dystrophy (a muscle disorder); and Kennedy's disease (a nerve problem)—had been discovered only in the year preceding the meeting and have been found to be similar. The mutations arise because of the instability of a portion of the DNA in the genes for each disorder. The gene in an affected individual is larger than in an unaffected person, and it continues to enlarge as it is passed on to the next generation. In addition to scientists investigating these disorders, the meeting was attended by scientists who have been working on genome instability in other organisms such as bacteria and yeast in the hope that common themes would be discernible.

Baring Brothers/Cold Spring Harbor Laboratory Meeting

The seventh of these annual meetings, funded initially by Shearson Lehman and more recently by Baring Brothers of London, was held in October 1992. These meetings comprise what has become one of our longest-lived series, so it was appropriate that the 1992 meeting was on **Aging**. The range of topics covered was broad, from the social and economic consequences of our aging population, through basic mechanisms of aging, to Alzheimer's disease. Once again, a group of world-class scientists discussed their latest research, and it was especially pleasing to have Carol Greider present her research on telomere shortening in aging cells. The executives were leaders in the worlds of biotechnology business and investment, and it is clear that this meeting has become a prestigious event.

Databases for Forensic DNA Fingerprinting

We held a meeting at Banbury Center in 1988 on DNA and Forensic Science that examined the new techniques of DNA "fingerprinting" that were just then being taken up in the United States. Although many of the issues raised at that meeting are resolved only partially, implementation of DNA typing has proceeded apace. One problem of tremendous practical consequence concerns the development of databases for storing DNA-typing information, in particular allowing for future technical developments and ensuring the security of such databases. Agencies in New York State are proceeding cautiously and carefully, and this meeting **DNA Forensic Fingerprinting** was held to explore the requirements of these agencies. In addition, representatives of the U.S. Federal Bureau of Investigation and the law enforcement agencies of Florida, Minnesota, and Virginia described their systems. The meeting ended with a discussion of a pilot project to develop a database for New York State.

Human Genome and Genetic Analysis Workshops

Banbury Center and the DNA Learning Center held the second and third in the current series of these workshops sponsored by the Department of Energy in 1992. The format followed that established with the first workshop, namely, lectures on the basics of genetics, talks by invited speakers at the Banbury Center, and practical laboratories at the DNA Learning Center. The presentations by the invited speakers were uniformly excellent and the participants welcomed the opportunity to spend time informally with scientists, genetic counselors, and bioethicists, subjecting them to merciless questioning. An example of the topicality of these workshops has been the presentation of talks on gene therapy by Dr. Kenneth Culver. The first gene therapy treatments were begun in September 1990 on children with a disorder called adenosine deaminase deficiency. We were delighted to welcome one of these children and her family to the workshop in February.

Science Policy Meetings

Banbury Center's role as a meeting place to discuss science policy began some 5 years ago with an influential meeting on scientific misconduct. This year, two fascinating meetings dealt with issues that affect the financing of scientific research. The first meeting, with the rather prosaic title **Indirect Costs and the Independent Research Institute**, was anything but dull. Indirect costs are those expenses relating to the operating costs of an institution that are charged to a grant, in addition to the direct costs of doing the research. Indirect costs can be substantial, and the ire of the Oversight and Investigations Subcommittee of the House Committee on Energy and Commerce was aroused when it appeared that some universities were charging inappropriate items to indirect costs. As a consequence, indirect cost rates for universities came under close scrutiny and revised rates are still being negotiated. Independent research institutions like Cold Spring Harbor Laboratory are governed by a separate set of rules, but it seems clear that these rules are also likely to be revised with potentially catastrophic financial consequences for the independent research institutes. So we decided to hold this meeting to review the current state of indirect cost

charges and to assess what changes were in store and what their consequences might be.

The second policy meeting discussed a topic of immense importance, namely, research in the neurosciences, including basic research in neurobiology and neurology. President Bush issued a proclamation declaring the 1990s the Decade of the Brain. However, relatively little seems to have come from this initiative and there seems to be an absence of leadership and coordination in exploiting this proclamation. The Charles A. Dana Foundation is developing a strong interest in medical neuroscience, and the Foundation provided funding for a meeting entitled **Funding for the Decade of the Brain**. The meeting brought together the leading players from the National Institutes of Health, the various Foundations, concerned with neuroscience, and some of the leading neuroscientists to determine what could be done. Many such meetings end on a high note, but their impact dissipates once the participants have dispersed; there is every reason to believe that this will not be the fate of this meeting. With the Dana Foundation playing the role of midwife, the meeting will give birth to a movement that will make the first decade of the 21st century (if not the 1990s) the Decade of the Brain.

Charles A. Dana Foundation and Manic-Depressive Illness

An event of great significance for the Laboratory and for the Banbury Center was the award of a grant from the Charles A. Dana Foundation for a program to promote research on manic-depressive illness. This program encompasses work at the Johns Hopkins Medical School, Stanford University Medical School, and Cold Spring Harbor Laboratory. Our part of the program, to be based at Banbury, will involve establishing a database that includes information on all aspects of manic-depressive illness and can be accessed by all research workers; this will be developed in collaboration with Tom Marr's group. In addition, we will develop a meetings and courses program as well as a public information program. This work will be a natural extension of Banbury's work on human genetics and science education. We expect that a program director will be appointed in the spring of 1993 and that the program will begin in earnest during the summer.

Other Meetings

As in previous years, we have made Banbury Center available to other groups. The Lloyd Harbor Seminar Series provides an opportunity to learn something of what goes on in the institutions that have their homes in Lloyd Harbor Village, as well as providing a venue to hear the interesting experiences of our neighbors. The Boards of a number of local community groups held their annual meetings here, including the Lloyd Harbor Historical Society, the Lloyd Harbor Conservation Board Society, and Huntington Hospital. In addition, the Cold Spring Harbor School District held a meeting here and the faculty of West Side school came for a pre-school conference.

Funding

The Corporate Sponsors Program continues to be the foundation on which the Banbury Center meetings program is built; six of our meetings in 1992 were

funded by this Program: **Phage Display: Engineering and Selecting Proteins; Molecular Mechanisms of Viral Latent Infections; Oligonucleotide Manipulation of Gene Expression: Its Therapeutic Potential; Control of HIV Gene Expression; Superantigens and Antigenic Presentation;** and **Mechanisms of Neuronal Survival: The Action of Neurotrophic Factors.** In addition, a number of other companies contributed to meetings during the year. For example, Repligen Corporation, Hoffmann-La Roche, and Merck Research Laboratories contributed to the **Control of HIV Gene Expression** meeting. Repligen Corporation together with Abbott Laboratories contributed to the **Constructing Organisms** meeting. However, private foundations continue to be major supporters of our program. I have already discussed the extraordinary support that the Alfred P. Sloan Foundation has provided during the past 14 years. The importance of such long-term support to an institution such as the Banbury Center cannot be overemphasized.

We continue to search for a replacement. There was not time in 1992 to hold a meeting funded by the William Stamps Farish Fund, but the first meeting of 1993 will be on the **Polygenic Basis of Cancer**. Here again, the 3-year support of this foundation helps provide stability for our program. The Wellcome Trust joined with us in holding the **Constructing Organisms** meeting, and the Charles A. Dana Foundation supported what we hope and expect will turn out to be a historic meeting: **Funding for the Decade of the Brain.**

Acknowledgments

1992 was an exceptionally busy year for the Center, especially during the fall when we had ten meetings and were also planning and organizing the spring 1993 meetings. Bea Toliver and Ellie Sidorenko in the Banbury Center office dealt with the huge influx of participants and coped with the flood of queries, phone calls, and faxes that accompanies every meeting. Katya Davey, hostess at Robertson House, looked after the participants with her customary panache.

Looking Forward to 1993

To begin by looking back, 1992 marked the end of my fifth year at the Banbury Center, and in that period, we have held 75 meetings with some 2500 participants. One of the great pleasures and virtues of the Banbury Center is that the topics of the meetings range so widely and that the participants are equally varied in their interests and backgrounds. Meetings and participants are unified in their commitment to excellence and all of these factors make Banbury Center a unique facility. As usual, the 1993 program is still being developed at this time, but it will provide the same variety of interests and live up to those same standards, with meetings on cancer, gene therapy, neurobiology, cell death, Lyme disease, and science policy. We will begin a new relationship with Helix Partners, which will fund meetings on the science underlying important biotechnological advances, and there will be the new developments on manic-depressive illness just described. One final acknowledgment is necessary. The work of the Banbury Center would not be possible without the support of Jim Watson and the entire Laboratory. My thanks, and those of everyone who comes to Banbury Center, go to all who work on the other side of the Harbor.

Jan A. Witkowski

MEETINGS

Congressional/Science Journalists Workshop on the Commercialization of Biology

January 24–January 26

FUNDED BY

Alfred P. Sloan Foundation

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

C. van Horn, U.S. Patent and Trademarks Office, Arlington, Virginia: Mechanics of obtaining a patent.
R. Merges, Boston University Law School, Massachusetts: Comparative studies of patenting in biology and other sciences.
J. Barton, Stanford University Law School, California: Special issues in biotechnology patenting.
G. Rathmann, ICOS, Bothell, Washington: Intellectual property and the development of biotechnology.

SESSION 2

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Isolation of DNA.

D. Botstein, R. Merges, R. Charrow

SESSION 3

D. Botstein, Department of Genetics, Stanford University School of Medicine, California: Materials and data sharing between scientists: The effects of commercialization.
R. Adler, Office of Technology Transfer, National Institutes of Health, Bethesda, Maryland: NIH's responsibilities and role in developing scientific discoveries.
A.L. Beaudet, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas: Impact of commercial considerations on the use of DNA-based diagnosis of genetic diseases.

SESSION 4

L.L. Nelsen, Technology Licensing Office, Massachusetts Institute of Technology, Cambridge: Why and how do academic institutions exploit the intellectual property of their scientists?
H. Edgar, Columbia University School of Law, New York, New York: Conflicts of interest in the exploitation of intellectual property.
R.P. Charrow, Crowell & Moring, Washington, D.C.: Summary of issues.

K. Hudson

Phage Display

April 4–April 7

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

G. Smith, University of Missouri, Columbia
M. Zoller, Genentech, Inc., South San Francisco, California

SESSION 1

P. Model, The Rockefeller University, New York, New York: Filamentous phage assembly.
G. Smith, University of Missouri, Columbia: Filamentous fusion and phage.

SESSION 2: Peptides on Phage

W.J. Dower, Affymax Research Institute, Palo Alto, California: Small peptides that mimic conformation-dependent epitopes: Isolation from phage display libraries.
G. Cesareni, University of Tor Vergata, Rome, Italy: Peptide libraries presented by the major coat protein of filamentous phages.
J. Scott, The Scripps Research Institute, La Jolla, California: Performance of a hexapeptide epitope library.
R.A. Houghten, Torrey Pines Institute for Molecular Studies, San Diego, California: The use of synthetic peptide combinatorial libraries for drug discovery and basic research.

SESSION 3: Methods and Alternative Displays

J.A. Wells, Genentech, Inc., South San Francisco, California: Applications of phage display technology for high-affinity and receptor selective hormone analogs.
W. Mandecki, Abbott Laboratories, Abbott Park, Illinois: Strategies for evolving novel proteins.
J.A. Sorge, Stratagene, La Jolla, California: λ13ZAP: Filamentous surface expression with the efficiency of λ packaging.
W. Szybalski, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison: Phage λ vectors.
M. Uhlen, Royal Institute of Technology, Stockholm, Sweden: Display of recombinant proteins on the surface of gram-positive bacteria.

SESSION 4: Antibodies on Phage

C.F. Barbas, The Scripps Research Institute, La Jolla, California: Combinatorial antibody libraries on the surface of phage: Opportunities in therapy and antibody engineering.
D. Burton, The Scripps Research Institute, La Jolla, California: Phage surface antibody libraries to select anti-viral antibodies.
D.J. Chiswell, Cambridge Antibody Technology Limited, United Kingdom: Phage display of functional protein domains: Isolation and improvement of antibodies.
L.J. Garrard, Genentech, Inc., South San Francisco, California: Generation of and selection from Fab phage display libraries.
M. Little, German Cancer Research Center, Heidelberg, Germany: Surface expression of antibodies on phagemid particles.

SESSION 5: Other Proteins on Phage

M. Zoller, Genentech, Inc., South San Francisco, California: Phage display and mutagenesis of human tumor necrosis factor.
C.S. Craik, University of California, San Francisco: Trypsin phage: Display of a proteolytic enzyme.
C. Hession, Biogen, Cambridge, Massachusetts: Phage display of VCAM-1 domains: Epitope mapping of VCAM-1 antibodies.
R.C. Ladner, Protein Engineering Corp., Cambridge, Massachusetts: Engineering neutrophil elastase inhibitors.
H. Lowman, Genentech, Inc., South San Francisco, California: Selection of variants of human growth hormones from random libraries using monovalent phage display.
J. Winter, Sandoz Crop Protection, Palo Alto, California: A model system for surrogate receptor evaluation.

SESSION 6: Peptides on Phage

R. Cortese, IRBM, Rome, Italy: Construction of epitope libraries.
V.A. Petrenko, Research and Technology Institute, Berdsk, Russia: Inserting foreign peptides into the major coat protein of bacteriophage M13.
R. Hoess, The Du Pont Merck Pharmaceutical Company, Wilmington, Delaware: Using constrained peptide libraries for epitope mapping and receptor binding.
R.N. Perham, University of Cambridge, United Kingdom: Display of foreign peptides on the surface of engineered bacteriophage fd: Immunological and other properties.
B. Malcolm, Chiron Corporation, Emeryville, California: Simplified construction and assessment of peptide libraries in bacteriophage.

M. Zoller, G. Smith, Guest, R. Houghten

Workshop on Human Genetics and Genome Analysis

February 23–February 26

FUNDED BY

Office of Health and Environmental Research, U.S. Department of Energy

ARRANGED BY

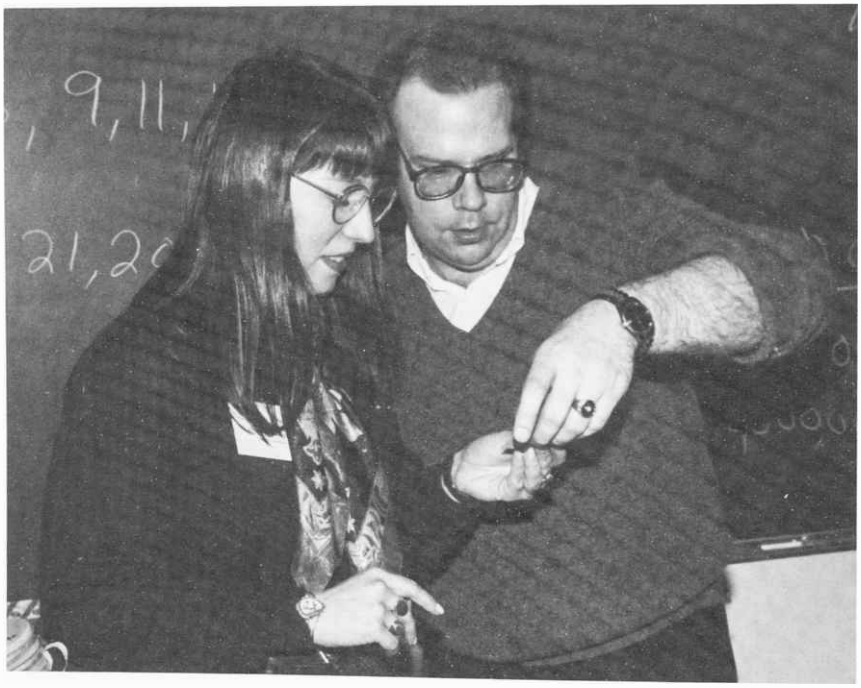
M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Mendelian view of the gene.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: Modern view of the gene.
T.H. Murray, Case Western Reserve University School of Medicine, Cleveland, Ohio: Ethical implications of human molecular genetics.

SESSION 2

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: DNA restriction enzymes and restriction mapping.



D. Erickson, M. Bloom

SESSION 3

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Cloning genes.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: Human molecular genetics and inherited disorders.

SESSION 4

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Transformation of *E. coli* with plasmid DNA.
M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Human DNA fingerprinting by polymerase chain reaction.

SESSION 5

E. Fearon, Johns Hopkins University School of Medicine, Baltimore, Maryland: Analyzing the molecular genetics of cancer.
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory results: Transformation of *E. coli* with plasmid DNA.
M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory results: DNA fingerprinting by polymerase chain reaction.

SESSION 6

K. Culver, National Institutes of Health, Bethesda, Maryland: The first human gene therapy trials.
J.D. Watson, National Center for Human Genome Research, Bethesda, Maryland; Cold Spring Harbor Laboratory, New York: The Human Genome Project.

K. Culver, D. Micklos, S. Cutshall

Congressional/Science Journalists Workshop on Occupational and Environmental Health

April 10–April 12

FUNDED BY

Alfred P. Sloan Foundation

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

P. Landrigan, Mt. Sinai Medical School, New York, New York: How serious is the problem and what are the economic consequences?

N.A. Ashford, Center for Technology, Policy and Industrial Development, Massachusetts Institute of Technology, Cambridge: Legal and regulatory issues in environmental and occupational health.

B.D. Goldstein, Environmental and Occupational Health Sciences Institute, Piscataway, New Jersey: Physicians: Environmental and occupational health.

SESSION 2

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: DNA experiment.

SESSION 3

K. Rest, Occupational Health Program, University of Massachusetts Medical Center, Amherst: Ethical aspects of occupational and environmental health.

F.W. Mirer, International Union, United Automobile Workers, Detroit, Michigan: A practical approach to preventing occupational injury and illness.

D.P. Rall, Washington, D.C.: What can be done? The responsibilities and activities of federal agencies.

G. Ellis, J. Witkowski

D. Liskowski, R. Borchelt, D. Vogt

Indirect Costs and the Independent Research Institute

April 30–May 1

ARRANGED BY

J.D. Watson, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Indirect Costs At Research Institutions

Chairperson: M.F. Singer, Carnegie Institution of Washington, D.C.

M.F. Singer, Carnegie Institution of Washington, D.C.: Introductory remarks
W. Keen, Cold Spring Harbor Laboratory, New York: Summary and review of questionnaire.

SESSION 2: Audits

Chairperson: F.J. McKay, Fox Chase Cancer Center, Philadelphia, Pennsylvania

J. O'Neill, KPMG Peat Marwick, Washington, D.C.: Audits of indirect costs at universities.
R.D. Leedy, Fox Chase Cancer Center, Philadelphia, Pennsylvania, and L.A. Keinath, The Wistar Institute, Philadelphia, Pennsylvania: Audits of indirect costs at research institutions.

SESSION 3: Perspectives on Washington

Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

M.F. Singer, Carnegie Institution of Washington, D.C.: A view from the NIH Subcommittee on Indirect Costs.
J. Pratt, The Whitehead Institute, Cambridge, Massachusetts: Congressional players and legislative activities.

SESSION 4: Future Strategies and General Discussion

Chairperson: G.M. Browne, Cold Spring Harbor Laboratory, New York

G. Schiff, James N. Gamble Institute of Medical Research, Cincinnati, Ohio: Role of AIRI.

Molecular Mechanisms of Viral Latent Infections

July 6–July 9

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

B. Roizman, University of Chicago, Illinois

SESSION 1: Herpesviruses I

Chairperson: B. Roizman, University of Chicago, Illinois

B. Roizman, University of Chicago, Illinois: Introduction.
E. Kieff, Harvard Medical School, Boston, Massachusetts: EBV latency.
G.I. Miller, Jr., Yale University School of Medicine, New Haven, Connecticut: Mechanism of the ZEBRA protein in the switch between latency and lytic replication of EBV.
P. Lai, Tampa Bay Research Institute, St. Petersburg, Florida: Cellular proteins that bind to ori-p sequence of EBV genome.
E.S. Mocarski, Stanford University School of Medicine, California: CMV latency.

SESSION 2: Herpesviruses II

Chairperson: E. Kieff, Harvard Medical School, Boston, Massachusetts

N. Frenkel, Tel Aviv University, Ramat Aviv, Israel: Latency and reactivation of human herpesviruses 6 and 7: In vivo and in culture.
A.Y. Sears, University of Chicago, Illinois: Function of host-specific origins of viral DNA replication in HSV latency.
J.G. Stevens, University of California, Los Angeles: HSV genetic expression during establishment, maintenance, and reactivation from latent infection.
J. Hay, State University of New York, Buffalo, School of Medicine: Expression of varicella zoster virus genes in neural tissues.

SESSION 3: Papillomaviruses

Chairperson: H.S. Ginsberg, Columbia University College of Physicians & Surgeons, New York, New York

H. zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Germany: Host cell control of papillomavirus genome persistence.

S. Vande Pol, National Cancer Institute, Bethesda, Maryland: The bovine papillomaviruses constitutive enhancer is essential for transformation, DNA replication, and the maintenance of latency.

L. Laimins, University of Chicago, Illinois: Regulation of human papillomavirus life cycle differentiating epithelium.

SESSION 4: RNA Viruses I

Chairperson: I.S.Y. Chen, University of California, Los Angeles, School of Medicine

I.S.Y. Chen, University of California, Los Angeles, School of Medicine: Incomplete reverse transcription as a mechanism for HIV-1 low-level persistence in T cells.

B.R. Cullen, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina: Role of REV in lentiviral latency.

SESSION 5: RNA Viruses II

Chairperson: I.S.Y. Chen, University of California, Los Angeles, School of Medicine

W. Greene, University of California, San Francisco: Molecular analysis of HIV-1 "latency": Role of cellular transcription factors in viral activation.

A.T. Haase, University of Minnesota, Minneapolis: New experimental tools for the analysis of viral latency.

M. Stevenson, University of Nebraska Medical Center, Omaha: Preintegration events influencing HIV-1 latency.

SESSION 6: Other RNA Viruses

Chairperson: R.M. Chanock, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

J.C. de la Torre, The Scripps Research Institute, La Jolla, California: Disturbances in host's differentiated functions caused by a noncytolytic virus.

M.L. Nibert, Harvard Medical School, Boston, Massachusetts: Persistent infections of murine L cells with mammalian reoviruses: Coevolution of cells and viruses during maintenance of persistent infection involves a specific early step in the infectious cycle.

SESSION 7: AAV; Adenoviruses

Chairperson: K.I. Berns, Cornell University Medical College, New York, New York

K.I. Berns, Cornell University Medical College, New York, New York: Integration and rescue of AAV from the integrated site.

R.J. Samulski, University of Pittsburgh, Pennsylvania: AAV latent infection.

H.S. Ginsberg, Columbia University College of Physicians & Surgeons, New York, New York: "How adenoviruses persist."

W.S.M. Wold, St. Louis University Medical Center, Missouri: Adenovirus proteins that counteract CTL and TNF/macrophage-mediated immunosurveillance.

Oligonucleotide Manipulation of Gene Expression: Its Therapeutic Potential

October 13–October 16

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

M. Matteucci, Gilead Sciences, Inc., Foster City, California

SESSION 1: Mechanistic, Tissue Culture, and In Vivo Antisense Results I

Chairperson: M. Matteucci, Gilead Sciences, Inc., Foster City, California

L. Neckers, National Cancer Institute, Bethesda, Maryland: In vivo continuous intrathecal infusion of oligonucleotides: Pharmacokinetics and efficacy.

G. Zon, Applied Biosystems, Inc., Foster City, California: Recent investigations of antisense phosphorothioate oligodeoxynucleotides.

Y.-C. Cheng, Yale University School of Medicine, New Haven, Connecticut: Cellular and molecular pharmacology of phosphorothioate oligodeoxynucleotides.

C. Stein, Columbia University College of Physicians & Surgeons, New York, New York: Pathways of oligonucleotide internalization in hematopoietic cell lines.

R.W. Wagner, Gilead Sciences, Inc., Foster City, California: Understanding hurdles in oligonucleotide antisense development.

B. Lebleu, Universite Montpellier II, Montpellier Cedex, France: Cell targeting, transmembrane passage, and intracellular distribution of antisense oligonucleotides.

SESSION 2: Mechanistic, Tissue Culture, and In Vivo Antisense Results II

Chairperson: P.S. Miller, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland

S. Agrawal, Hybridon, Inc. Worcester, Massachusetts: Oligonucleotide phosphorothioates as antiviral agents.

R. Juliano, University of North Carolina School of Medicine, Chapel Hill: Cellular uptake and intracellular distribution of chemically modified oligonucleotides.

L. Arnold, Genta Inc., San Diego, California: Development of antisense therapeutic agents.

D.J. Chin, Agouron Institute, La Jolla, California: Hybrid complexes in living cells studied by fluorescence energy transfer.

J.-J. Toulme, Universite de Bordeaux II, Bordeaux Cedex, France: Efficiency and selectivity of RNase-H-mediated inhibition of translation and reverse transcription by antisense oligonucleotides.

A.M. Krieg, University of Iowa, Iowa City: Effects of oligonucleotide modification on cell binding, uptake, and antisense efficacy.

SESSION 3: New Structural Modifications of Oligonucleotides I

Chairperson: M.H. Caruthers, University of Colorado, Boulder

P.S. Miller, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland: Interaction of base analogs with dsDNA.

C.K. Mirabelli, ISIS Pharmaceuticals, Carlsbad, California: Pharmacokinetics of chemically modified oligonucleotides: Impact on therapeutic uses.

J.A. Walder, University of Iowa, Iowa City: Synthesis and therapeutic applications of new achiral oligonucleotide analogs.

G. Trainor, The Du Pont Merck Pharmaceutical Company, Wilmington, Delaware: Oligonucleotide analogs with sulfamate and sulfamid internucleotide linkages.

H.E. Moser, Ciba-Geigy AG, Basel, Switzerland: Carbocyclic oligonucleotides: Synthesis and properties.

C. Mirabelli, E. Saison-Behmoaras

SESSION 4: New Structural Modifications of Oligonucleotides II

Chairperson: J.A. Walder, University of Iowa, Iowa City

P.E. Nielsen, The Panum Institute, Copenhagen, Denmark: Sequence-specific DNA recognition by peptide nucleic acid chimera.

L.E. Babiss, Glaxo, Inc., Research Triangle Park, North Carolina: Antisense and antigenic potentials of peptide nucleic acid oligomers.

M.H. Caruthers, University of Colorado, Boulder: Synthesis, biochemistry, and biological applications of new polynucleotide analogs.

J.H. van Boom, State University Leiden, The Netherlands: A study directed toward the preparation and hybridization potential of DNA fragments containing internucleosidic methylene acetal bonds at predetermined positions.

E. Saison-Behmoaras, INSERM/CRS, Paris, France: Selective inhibition of mutation Ha-*ras* expression by antisense oligonucleotides.

SESSION 5: Triple Helix Results

Chairperson: H.E. Moser, Ciba-Geigy AG, Basel, Switzerland

M. Matteucci, Gilead Sciences, Inc., Foster City, California: Phosphodiester analogs of oligonucleotides.

M.E. Hogan, Baylor College of Medicine, The Woodlands, Texas: Triplex forming oligonucleotides.

J. Klysik, Texas A&M University, Houston: DNA triplexes.

L.-S. Kan, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland: How to increase the stability of triplex formation.

S.F. Singleton, California Institute of Technology, Pasadena: An analysis of the energetics of triple helix formation using quantitative affinity cleavage titration.

D.J. Patel, Columbia University College of Physicians & Surgeons, New York, New York: Solution structure of Y-RY and R-RY DNA triplexes.

DNA Repeats and Human Gene Mutations

October 18–October 21

FUNDED BY

U.S. Department of Energy

ARRANGED BY

M. Radman, Institut Jacques Monod, Paris, France
S.T. Warren, Howard Hughes Medical Institute, Emory University School of Medicine, Atlanta, Georgia
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Human Genome: Fragile X

Chairperson: M. Radman, Institut Jacques Monod, Paris, France

S.L. Sherman, Emory University, Atlanta, Georgia: Population dynamics of the fragile-X mutation.

D.L. Nelson, Baylor College of Medicine, Houston, Texas: Structure of the CGG repeats in FMR-1.

R.I. Richards, Adelaide Children's Hospital, North Adelaide, South Australia: Trinucleotide repeats in human genes: Inclusion by chance or necessity—Common properties of disorders caused by dynamic mutation.

J.-L. Mandel, LGME/CNRS, INSERM U184, Strasbourg, France: Phenomenology of mutation and methylation in fragile-X syndrome.

K.E. Davies, John Radcliffe Hospital, Oxford, United Kingdom: Methylation, fragile sites, and instability.

SESSION 2: Myotonic Dystrophy

Chairperson: S.T. Warren, Emory University School of Medicine, Atlanta, Georgia

D. Shaw, University of Wales College of Medicine, Cardiff: Myotonic dystrophy—Analysis of the mutation and speculations on nature of gene product.

K. Johnson, Charing Cross and Westminster Medical School, London, United Kingdom: Characterization of the CTG repeat in myotonic dystrophy patients.

SESSION 3: Spinal Bulbar Muscular Atrophy

Chairperson: S.T. Warren, Emory University School of Medicine, Atlanta, Georgia

K.H. Fischbeck, University of Pennsylvania Medical School, Philadelphia: The molecular genetics of X-linked spinal and bulbar muscular atrophy.

J.-L. Mandel, LGME/CNRS, INSERM U184, Strasbourg, France: Instability in SBMA.

SESSION 4: Other Sequences

Chairperson: D.L. Nelson, Baylor College of Medicine, Houston, Texas

R.M. Cawthon, University of Utah Medical Center, Salt Lake City: Mutations in two tumor suppressor genes: Neurofibromatosis 1 and adenomatous polyposis coli.

S.S. Sommer, Mayo Clinic/Foundation, Rochester, Minnesota: The underlying pattern of spontaneous human germ-line mutation: Lessons from the factor IX gene.

M. Schalling, Massachusetts Institute of Technology, Cambridge: Detection of novel expanded repeats and expression of repeat containing genes.

S.T. Warren, Emory University School of Medicine, Atlanta, Georgia: Repeat sequences in other genes.

SESSION 5: Data from Other Genomes

Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory

T.D. Petes, University of North Carolina, Chapel Hill: Genetic control of the stability of simple repetitive yeast DNA sequences.

J. Armour, University of Leicester, United Kingdom: Repeat unit turnover at human minisatellite loci.

M. Radman, Institut Jacques Monod, Paris, France: Homologous DNA interactions in the evolution of gene and chromosome structure.

M.A. Resnick, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina: Genome stability: The interaction between recombination, repair, and cell signaling.

R.D. Wells, Texas A&M University, Houston: Unusual DNA structures and repeat sequences.

R.R. Sinden, Texas A&M University, Houston: The impact of DNA secondary structure on mutations and preferential mutagenesis of leading and lagging strands.

M.S. Fox, Massachusetts Institute of Technology, Cambridge: Mismatch repair and mutation avoidance in *E. coli*.

R.M. Liskay, Yale University School of Medicine, New Haven, Connecticut: Multiple homologs of a DNA mismatch repair gene (*MutL*) in mammals and yeast.

SESSION 6: Generation of Sequence Polymorphisms

Chairperson: K.E. Davies, John Radcliffe Hospital, Oxford, United Kingdom

E.U. Selker, University of Oregon, Eugene: Inactivation of duplicated genes in fungi.

G. Faugeron, University of Paris-South, Orsay, France: Cytosine methylation and gene silencing triggered by DNA repeats.

P. Kourilsky, INSERM U277, Paris, France: The extensive polymorphism of the major transplantation antigens and its possible relationship with the CpG content of MHC genes.

T.P. Yang, University of Florida College of Medicine, Gainesville: High-resolution methylation analysis of the FMR-1 repeat by genomic sequencing.

SESSION 7: Recombination in Mammalian Genes

Chairperson: K.E. Davies, John Radcliffe Hospital, Oxford, United Kingdom

R.S. Kucherlapati, Albert Einstein College of Medicine, Bronx, New York: Manipulating the mammalian genome through homologous recombination.

H. Eisen, Fred Hutchinson Cancer Research Center, Seattle, Washington: Use of pseudogenes and recombination in the generation of diversity and ordered gene expression.

J.-C. Weill, Institut Necker, Paris, France: Molecular mechanisms involved in the generation of antibody diversity (gene conversion, somatic mutation).

K. Davies

E. Selker

Control of HIV Gene Expression

October 24–October 27

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program, Repligen Corporation, Merck Research Laboratories, and Hoffmann-La Roche Inc.

ARRANGED BY

B.R. Cullen, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina
R. Franza, Cold Spring Harbor Laboratory, New York
F. Wong-Staal, University of California, San Diego, School of Medicine, La Jolla

SESSION 1: Infection and Integration

Chairperson: R. Franza, Cold Spring Harbor Laboratory, New York

J.P. Moore, The Aaron Diamond AIDS Research Center, New York University School of Medicine, New York: Envelope glycoprotein of HIV-1: Roles of HIV-1 entry into $CD4^+$ cells and neutralization thereof.
D.R. Littman, University of California, San Francisco: Role of CD4 in HIV entry and pathogenesis.
H.E. Varmus, University of California, San Francisco, School of Medicine: Retroviral integration.
M. Stevenson, University of Nebraska Medical Center, Omaha: Nuclear import of HIV-1 preintegration complexes: Cell cycle dependence.
M.A. Martin, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland: Cell-to-cell virus spread.
M. Emerman, Fred Hutchinson Cancer Research Center, Seattle, Washington: HIV infection of nondividing cells.

SESSION 2: Cellular Factors

Chairperson: H.E. Varmus, University of California, San Francisco, School of Medicine

R. Franza, Cold Spring Harbor Laboratory, New York: Differential expression of KB-binding protein and their cellular associates.
A.S. Baldwin, University of North Carolina, Chapel Hill: Control of NF-κB activity.
P.A. Baeuerle, Genzentrum de LMU, Martinsried, Germany: Oxidative stress and the activation of NF-κB and HIV.
G.J. Nabel, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, Michigan: Role of NF-κB in HIV transcriptional initiation and virus replication.
W.C. Greene, Howard Hughes Medical Institute, Gladstone Institute of Virology and Immunology, San Francisco, California: NF-κB and the regulation of HIV-1 gene expression.
R.G. Roeder, The Rockefeller University, New York, New York: Role of cellular factors in basal and Tat-induced HIV transcription.
N. Hernandez, Cold Spring Harbor Laboratory, New York: Formation of short transcripts in the HIV-1 LTR.

SESSION 3: Tat and Rev I

Chairperson: F. Wong-Staal, University of California, San Diego, School of Medicine, La Jolla

B.R. Cullen, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina: Recent insights into HIV-Rev function.
R. Gaynor, University of Texas Southwestern Medical School, Dallas: HIV DNA and RNA binding proteins.
K.A. Jones, The Salk Institute, La Jolla, California: HIV-1 transcription: Tat, TAR, and cellular factors.
B.M. Peterlin, Howard Hughes Medical Institute, University of California, San Francisco: Tat and transcriptional regulation of HIV.
M.F. Laspia, Cold Spring Harbor Laboratory, New York: *Trans*-activation of transcription by Tat.
J. Karn, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom: Tat and rev: Chemistry of RNA binding and biological properties in vivo and in vitro.

D. Littman, B. Cullen

SESSION 4: Tat and Rev II

Chairperson: B.R. Cullen, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina

F. Wong-Staal, University of California, San Diego, School of Medicine, La Jolla: Role of NF_{RRE} in HIV replication and latency.

M. Zapp, University of Massachusetts Medical Center, Worcester: Mechanisms of Tat and Rev action.

A. Frankel, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts: Structural determinants of Tat and Rev RNA recognition.

T.G. Parslow, University of California, San Francisco: REV and REX.

J. Hauber, Sandoz Research Institute, Vienna, Austria: Functional analysis of the HIV-1 Rev *trans*-activator.

G.N. Pavlakis, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland: Rev protein counteracts inhibitory/instability sequences throughout the HIV-1 genome.

SESSION 5: Other Aspects

Chairperson: M.A. Martin, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

R.C. Desrosiers, Harvard Medical School, Southborough, Massachusetts: The importance of "nonessential" genes for AIDS pathogenesis.

J. Skowronski, Cold Spring Harbor Laboratory, New York: HIV-1 *nef* gene and T-cell activation.

H. Goettlinger, Dana-Farber Cancer Institute, Cambridge, Massachusetts: New insights regarding the function of the HIV-1 regulatory protein *vpu*.

M.H. Malim, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia: Inhibition of HIV-1 replication using transdominant mutants of the viral Rev *trans*-activator.

E. Gilboa, Sloan-Kettering Institute, New York, New York: Intracellular immunization against HIV using RNA decoys.

R. Desrosiers, J. Skowronski

K. Roeder, N. Hernandez

Baring Brothers/Cold Spring Harbor Laboratory Executive Conference on Aging

October 30–November 1

ARRANGED BY

J.D. Watson, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

J.D. Watson, Cold Spring Harbor Laboratory, New York: Introductory remarks.
R.N. Butler, The Mt. Sinai Medical Center, New York, New York: The longevity revolution.

SESSION 2

G.M. Martin, University of Washington School of Medicine, Seattle: Implications of evolutionary theory for human aging.
B.N. Ames, University of California, Berkeley: Mechanisms of aging: Oxidation.

SESSION 3

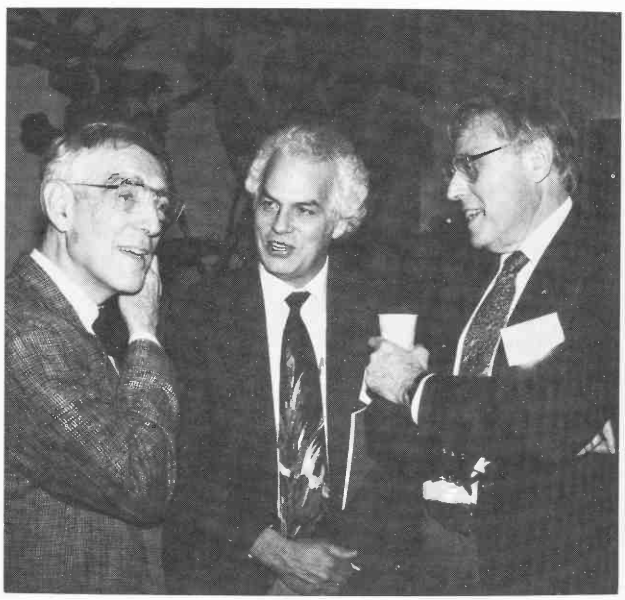
D. Micklos and M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: DNA fingerprinting by PCR.

SESSION 4

S.B. Prusiner, University of California, San Francisco, School of Medicine: Prions and neurodegenerative diseases.
D.L. Price, The Johns Hopkins University School of Medicine, Baltimore, Maryland: Neurotrophic factors and aging of the central nervous system.
C. Greider, Cold Spring Harbor Laboratory, New York: Exciting developments in aging research at Cold Spring Harbor.

SESSION 5

D.J. Selkoe, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts: Amyloid protein and the molecular mechanism of Alzheimer's disease.
A.D. Roses, Duke University Medical Center, Durham, North Carolina: Alzheimer's disease: Unraveling the genetics.
D. Micklos and M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: DNA fingerprinting results.
D.E. Redmond, Jr., Yale University School of Medicine, New Haven, Connecticut: Fetal tissue transplantation and Parkinson's disease.



B. Ames, S. Prusiner, G. Martin

Funding for the Decade of the Brain

November 4–November 6

FUNDED BY

Charles A. Dana Foundation

ARRANGED BY

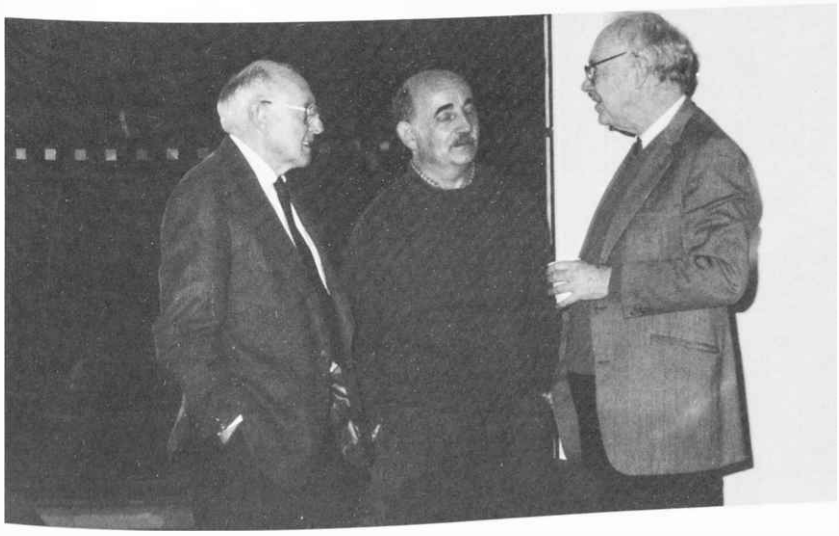
W.M. Cowan, Howard Hughes Medical Institute, Bethesda, Maryland
K.R. Jamison, Washington, D.C.
F. Plum, Cornell University Medical College, New York, New York
J.D. Watson, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: The Decade of the Brain: Impact on Research
Discussion Leader: H. Pardes, New York State Psychiatric Institute, New York

Review of the genesis, intent, and funding of the Proclamation on the Decade of the Brain.
L.L. Judd, University of California, San Diego, School of Medicine, La Jolla

How the National Institutes of Health have been able to respond.
M. Goldstein, National Institute of Neurological Diseases and Stroke, Bethesda, Maryland

Impact on the academic community.
R.C. Collins, University of California, Los Angeles
J.B. Martin, University of California, San Francisco, School of Medicine
S.J. Ryan, University of Southern California Medical School, Los Angeles
J.D. Barchas, University of California, Los Angeles, School of Medicine



W. M. Cowan, A. Aguayo, J. Watson

SESSION 2: The Decade of the Brain: Who Needs to be Persuaded of Its Importance?

Discussion Leader: G.M. McKhann, Johns Hopkins University, Baltimore, Maryland

(A) Are there useful models for the Battle for the Brain?

The war on cancer
J.D. Watson, Cold Spring Harbor Laboratory, New York

The Human Genome Project
R. Cook-Deegan, National Academy of Sciences, Washington, D.C.

Alzheimer's disease
N.G. Cavarocchi, CR Associates, Washington, D.C.

The scientific community; Congress; the media; advocacy groups
K.R. Jamison, Washington, D.C.

Reaching out to influential members of business and media
D. Mahoney, The Charles A. Dana Foundation, New York, New York

(B) Strategies for arousing the public's interest and enthusiasm

The fascination of knowing how the normal mind works
G.M. McKhann, Johns Hopkins University, Baltimore, Maryland

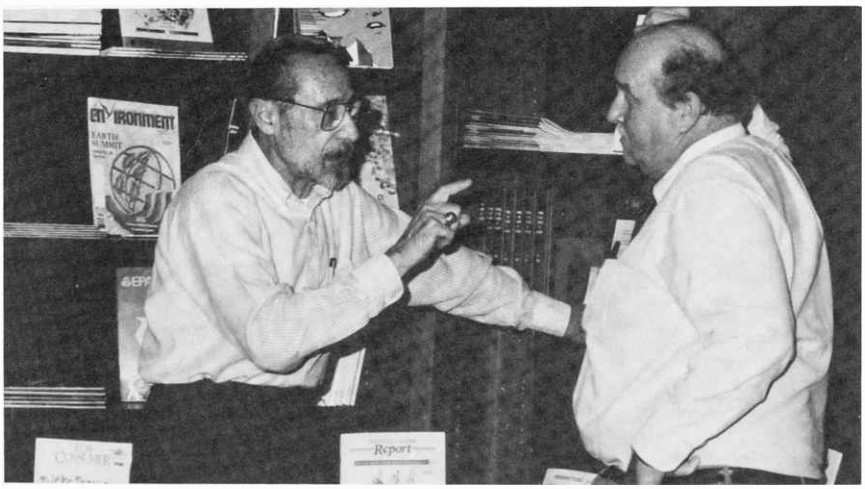
Targeting diseases
D.J. Selkoe, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts
E..M. Shooter, Stanford University School of Medicine, California

Economic arguments
R.J. Wyatt, National Institute of Mental Health Neuroscience Center at St. Elizabeth's, Washington, D.C.

SESSION 3: How to Reach Specific Groups?

Discussion Leader: R.M. Rose, The John D. & Catherine T. MacArthur Foundation, Chicago, Illinois

Advocacy groups and the scientific community
H. Pardes, New York State Psychiatric Institute, New York Foundations



M. Goldstein, B. Metheny

R.M. Rose, The John D. & Catherine T. MacArthur Foundation, Chicago, Illinois
D. Mahoney, The Charles A. Dana Foundation, New York, New York

Congress: Who are the key figures?
F.K. Goodwin, National Institute of Mental Health, Rockville, Maryland
B. Metheny, South Dartmouth, Massachusetts

SESSION 4: Determination of Specific Goals

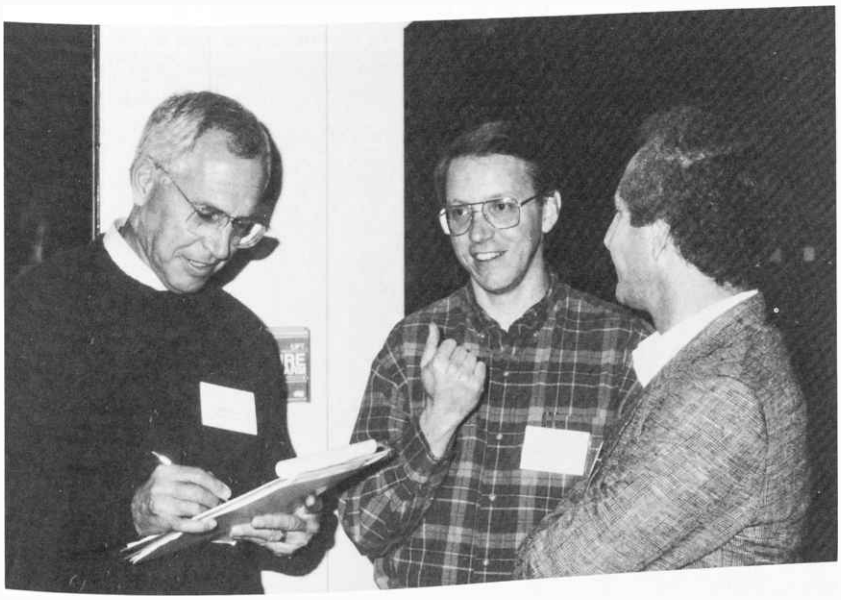
Discussion Leader: F. Plum, Cornell University Medical College, New York, New York

General Discussion:
Setting goals that have a high priority in terms of their inherent interest and importance and that have the potential for exciting public support for the whole of the Decade of the Brain program. This discussion emphasized (a) topics where funding would make a significant difference; (b) why a major effort should be made to achieve these goals; (c) how to ensure that the commitment to these goals is maintained; and (d) how to ensure that the progress made is used to further the aims of the project.

SESSION 5: Consolidation of Goals

Discussion Leader: W.M. Cowan, Howard Hughes Medical Institute, Bethesda, Maryland

General Discussion
HOW CAN THe efforts and results of the present meeting be used best?
What should be done by federal institutes, academic associations, and foundations to further the aims of the Decade of the Brain?
Can these efforts be coordinated?
What are the political dimensions?
What political strategies need to be pursued (lobbying?)?



R. Rose, R. Cook-Deegan

Superantigens and Antigenic Presentation

November 8–November 11

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

B.T. Huber, Tufts University School of Medicine, Boston, Massachusetts
E. Palmer, National Jewish Center, Denver, Colorado

SESSION 1: Bacterial SAG/MHC/TCR Interaction

Chairperson: R.J. Hodes, National Cancer Institute, Bethesda, Maryland

J.W. Kappler, Howard Hughes Medical Institute, National Jewish Center, Denver, Colorado: Structural features of superantigens.

S. Swaminathan, VA Medical Center, Pittsburgh, Pennsylvania: The crystal structure of staphylococcal enterotoxin B.

J.D. Fraser, University of Auckland School of Medicine, New Zealand: Structural studies of staphylococcal enterotoxins A and E. Functional domains involved in TcR and MHC binding.

D.R. Karp, University of Texas Southwestern Medical Center, Dallas: Distinct binding sites on HLA-DR for invariant chain and staphylococcal enterotoxins.

R.S. Geha, Children's Hospital, Boston, Massachusetts: Signal transduction via Ia (MHC class II) molecules.

N.S. Braunstein, Columbia University College of Physicians & Surgeons, New York, New York: T-cell recognition of MHC molecules.

SESSION 2: T-cell Interaction with SAG

Chairperson: E. Palmer, National Jewish Center, Denver, Colorado

S.R. Webb, The Scripps Research Institute, La Jolla, California: Mls antigens: Immunity and tolerance.

R. Abe, Naval Medical Research Institute, Bethesda, Maryland: Role of superantigens in T-cell receptor-mediated signaling and T cell repertoire selection.

D. Woodland, St. Jude Children's Research Hospital, Memphis, Tennessee: MHC-restricted recognition of superantigens.

M. Blackman, St. Jude Children's Research Hospital, Memphis, Tennessee: Role of the TCR α chain in recognition of superantigens.

E. Simpson, Clinical Research Centre, Harrow, Middlesex, United Kingdom: Effect of endogenous superantigens on T cell repertoire selection of transgenic mice.

K. Tomonari, Clinical Research Centre, Harrow, Middlesex, United Kingdom: Vβ repertoire and viral superantigens.

Discussion: R.J. Hodes, National Cancer Institute, Bethesda, Maryland and J. Sprent, The Scripps Research Institute, La Jolla, California

SESSION 3: Retroviral Biology

Chairperson: H. Diggelmann, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland

S.R. Ross, University of Illinois College of Medicine, Chicago: Endogenous superantigen expression protects mice against MMTV infection.

H. Acha-Orbea, Ludwig Institute for Cancer Research, Lausanne, Switzerland: Infectious minor lymphocyte stimulating antigens.

H.C. Morse, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland: Murine AIDS: Possible role of an MLV superantigen in pathogenesis.

P. Jolicoeur, Clinical Research Institute of Montreal, Quebec, Canada: Is Pr65 GAG protein of the murine AIDS virus a superantigen?

B. T. Huber, Tufts University School of Medicine, Boston, Massachusetts: Biology of MTV-7 SAG expression.

P.-A. Cazenave, Institut Pasteur, Paris, France: Polymorphism of TCR Vβ regions and clonal deletions in wild mouse populations.

SESSION 4: Control of MMTV Expression

Chairperson: A. Korman, Institut Pasteur, Paris, France

G.J. Thorbecke, New York University Medical Center, New York: MTV-encoded superantigen expression in B lymphoma cells in SJL mice as a stimulus for "reverse immunosurveillance."

J.S. Butel, Baylor College of Medicine, Houston, Texas: Characterization of the MMTV LTR ORF gene product.

R.B. Corley, Duke University Medical Center, Durham, North Carolina: Regulation of MMTV genes and gene products in cells of the B lineage.

W.H. Gunzburg, Institute of Molecular Virology, Neuherberg, Germany: MMTV-encoded *naf* and superantigen activities: Different functions of the same gene?

SESSION 5: Viral Superantigens in Humans

Chairperson: J. Sprent, The Scripps Research Institute, La Jolla, California

D.N. Posnett, Cornell University Medical College, New York, New York: Is HIV-1 associated with a superantigen?

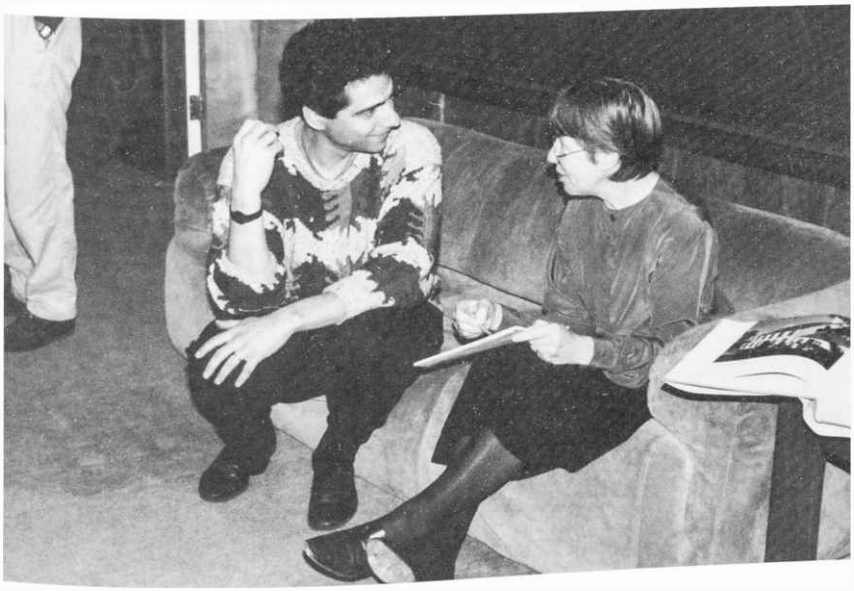
R.-P. Sekaly, Clinical Research Institute of Montreal, Quebec, Canada: Structure-function interaction of bacterial and viral superantigens with human T cells.

D. Primi, Conbiotec-Laboratory of Biotechnology, Brescia, Italy: Role of superantigens in human infections.

M. Lafon, Institut Pasteur, Paris, France: Evidence for a viral superantigen in humans: The rabies virus nucleocapsid.

B.L. Kotzin, National Jewish Center, Denver, Colorado: Superantigens in human autoimmune diseases.

P. Marrack, Howard Hughes Medical Institute, National Jewish Center, Denver, Colorado: Control of Vβ3 bearing cells in man.



E. Palmer, E. Simpson

Constructing Organisms

November 13–November 16

FUNDED BY

The Wellcome Trust, Repligen Corporation and Abbott Laboratories

ARRANGED BY

M. Bate, University of Cambridge, United Kingdom
A. Martinez-Arias, University of Cambridge, United Kingdom

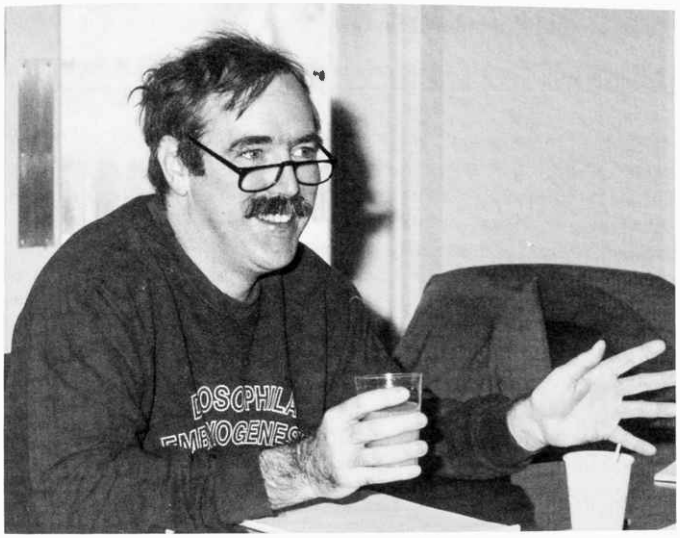
SESSION 1: Gene Expression and Allocation of Cell Fates in Early Embryos

E.H. Davidson, California Institute of Technology, Pasadena: Cell interaction and gene expression in the initial specification of lineage founder cells in the sea urchin embryo.
M. Fuller, Stanford University School of Medicine, California: Control of cell division and subcellular morphogenesis during spermatogenesis.
E. Wieschaus, Princeton University, New Jersey: Morphogenetic movements during gastrulation in *Drosophila*.
H. Jaeckle, Max-Planck-Institute of Biophysical Chemistry, Gottingen, Germany: Positional information: Complex interactions of transcriptional activators and repressors in *Drosophila*.
T. Doniach, University of California, San Francisco: Induction of anteroposterior neural pattern in *Xenopus*.
S. Cohen, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas: Allocation of the imaginal disc primordia in the *Drosophila* embryo.
G. Jurgens, University of Munich, Germany: Early events in apical-basal pattern formation in the *Arabidopsis* embryo.
N. Hopkins, Massachusetts Institute of Technology, Cambridge: Genetic approaches to early vertebrate development using zebrafish.

SESSION 2: Patterning the Nervous System

M. Bate, University of Cambridge, United Kingdom: Introduction.
T.M. Jessell, Howard Hughes Medical Institute, Columbia University, New York, New York: Signals that control neural cell pattern in vertebrates.
J.A. Campos-Ortega, University of Cologne, Germany: Mechanisms of early neurogenesis.
Y.-N. Jan, Howard Hughes Medical Institute, University of California, San Francisco: Mechanisms shared by neural development, sex determination, and oogenesis in *Drosophila*.
C.Q. Doe, University of Illinois, Urbana: The role of wingless/wrt-1 in *Drosophila* neural precursor formation and specification.
A. McMahon, Roche Institute of Molecular Biology, Nutley, New Jersey: Cell signalling in CNS development.

E. Macagno, N. Hopkins, K. Howard



E. Wieschaus

M.J. Stern, Yale University, New Haven, Connecticut: Conserved signaling systems controlling cell migration and cell fate determination in *C. elegans*.

E. Macagno, Columbia University, New York, New York: Factors affecting the assembly of the nervous system of the leech: Neuron-neuron and neuron-target interactions.

M.C. Raff, University College London, United Kingdom: Control of a neural cell death and survival.

D. Ready, Purdue University, West Lafayette, Indiana: The cytoskeleton in *Drosophila* eye development.

I.A. Meinertzhagen, Dalhousie University, Halifax, Nova Scotia: Dendritic retraction, sprouting, and reactive synaptogenesis: Evidence for growth factors in the lamina of *Drosophila*?

SESSION 3: Cell Behavior during Development

K. Howard, Roche Institute of Molecular Biology, Nutley, New Jersey: Introduction.

V. Hartenstein, University of California, Los Angeles: Embryonic development of the *Drosophila* head.

J.W. Fristrom, University of California, Berkeley: Functional requirements for changes in cell shape.

H. Skaer, University of Cambridge, United Kingdom: Cell interactions in Malpighian tubule development during *Drosophila* embryogenesis.

M. Ashburner, University of Cambridge, United Kingdom: Concluding remarks.

M. Raff, T. Jessell

Mechanisms of Neuronal Survival: The Action of Neurotrophic Factors

November 19–November 22

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

M. Furth, Regeneron Pharmaceuticals, Inc., Tarrytown, New York
R. McKay, Massachusetts Institute of Technology, Cambridge

SESSION 1: Themes and Variations–Neurotrophins, CNTF, and Beyond

Chairperson: M. Furth, Regeneron Pharmaceuticals, Inc., Tarrytown, New York

M. Furth, Regeneron Pharmaceuticals, Inc., Tarrytown, New York: Introductory remarks.
L.A. Greene, Columbia University College of Physicians & Surgeons, New York, New York: Neurotrophins and survival of cell bodies and axons.
G.D. Yancopoulos, Regeneron Pharmaceuticals, Inc., Tarrytown, New York: The receptors and signaling pathways that neurotrophic factors use to keep neurons alive.
S. Landis, Case Western Reserve University, Cleveland, Ohio: Regulation of neurotransmitter phenotypes by neurokines.

SESSION 2: Neurotrophins and Their Receptors

Chairperson: G.D. Yancopoulos, Regeneron Pharmaceuticals, Inc., Tarrytown, New York

B. Hampstead, Cornell University Medical College, New York, New York: Functions of NGF receptors.
M. Barbacid, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey: The *trk* family of neurotrophin receptors.
D.R. Kaplan, National Cancer Institute-Frederick Cancer Research and Development Center, Maryland: Neuronal signal transduction by *trk* receptors.
D. Soppet, National Cancer Institute-Frederick Cancer Research and Development Center, Maryland: Differential responses of *trk* family members to neurotrophins.
C. Ibanez, Karolinska Institute, Stockholm, Sweden: Structure-function relationships of neurotrophins and their receptors.
P. Barker, Stanford University School of Medicine, California: Tissue specific alternative splicing generates two isoforms of the *trk*A receptor
A. Rosenthal, Genentech, Inc., South San Francisco, California: NT-5, and beyond?

SESSION 3: Actions and Regulation of Neurotrophic Factors *In Vivo*

Chairperson: M.E. Hatten, Columbia University College of Physicians & Surgeons, New York, New York

R.M. Lindsay, Regeneron Pharmaceuticals, Inc., Tarrytown, New York: Neurotrophic factors from culture to the clinic.
K. Nikolics, Genentech, Inc. South San Francisco, California: Developmental regulation of neurotrophin receptors.
H. Persson, Karolinska Institute, Stockholm, Sweden: Regulation of neurotrophins and their receptors.
F. Hefti, University of Southern California, Los Angeles: Physiological role and neuroprotective actions of neurotrophins in the adult brain.
C. Gall, University of California, Irvine: Regulation of neurotrophin expression: Evidence for cellular specificity in the response properties of forebrain neurons.
F.H. Gage, University of California, San Diego, La Jolla: Cell survival and regeneration in the adult brain.
L. Williams, The Upjohn Company, Kalamazoo, Michigan: Effects of NGF on cholinergic transmission in the young and aged rat.

SESSION 4: Interactions of Neuronal Precursors and Neurotrophic Factors

Chairperson: S. Landis, Case Western Reserve University, Cleveland, Ohio

D.J. Anderson, Howard Hughes Medical Institute, California Institute of Technology, Pasadena: Acquisition of NGF-responsiveness and NGF-dependence by embryonic sympathetic neurons.
S. Weiss, University of Calgary, Alberta, Canada: Growth factor regulation of CNS stem cells and progeny *in vitro*.
M.E. Hatten, Columbia University College of Physicians & Surgeons, New York, New York: Control of cerebella granule cell neurogenesis and differentiation.
D. Lindholm, Max-Planck-Institute for Psychiatry, Munich, Germany: Neurotrophins in rat cerebellum: Action and regulation.
R. McKay, Massachusetts Institute of Technology, Cambridge: Neurotrophin responses in CNS precursor cells.

SESSION 5: Signal Transduction Mechanisms

Chairperson: M. Barbacid, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey

M.E. Greenberg, Harvard Medical School, Boston, Massachusetts: Neurotrophin regulation of gene expression during cell growth and differentiation.
H. Nawa, Cold Spring Harbor Laboratory, New York: Regulation of neuropeptide expression by BDNF.
T. Pawson, Mt. Sinai Hospital, Toronto, Ontario, Canada: SH2 and SH3 domains in signal transduction.
J. Schlessinger, New York University Medical Center, New York: Signal transduction by receptors that regulate tyrosine phosphorylation.
S. Green, University of Iowa, Iowa City: Convergence and specificity in intracellular signaling in PC12 cells.

M. E. Hatten, M. Greenberg

SESSION 6: Mechanisms of Neuronal Death

Chairperson: R. McKay, Massachusetts Institute of Technology, Cambridge

D. Hockenbery, Fred Hutchinson Cancer Research Center, Seattle, Washington: Regulation of cell death and the action of *bd-2*.
S. Estus, Washington University Medical School, St. Louis, Missouri: Studies of gene expression in neurons undergoing programmed cell death.
E.W. Rubel, University of Washington, Seattle: Afferent regulation of neuronal life and death: Plasticity of cytoplasmic organelles.
M.A. Bothwell, University of Washington, Seattle: Ribosome structural changes: An early event in neuronal cell death.
R. McKay, Massachusetts Institute of Technology, Cambridge: Concluding remarks.

Workshop on Human Genetics and Genome Analysis

December 6–December 9

FUNDED BY

Office of Health and Environmental Research, U.S. Department of Energy

ARRANGED BY

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Mendelian view of the gene.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: Modern view of the gene.
N. Press, University of California, Los Angeles, Medical Center: Population screening for genetic diseases.

J. Botkin, P. Ward

SESSION 2

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Using restriction enzymes and constructing chromosome maps.

SESSION 3

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Cloning genes.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: DNA diagnosis of human genetic diseases.
P. Ward, Baylor College of Medicine, Houston, Texas: Counseling for human genetic diseases.

SESSION 4

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Human DNA fingerprinting by polymerase chain reaction.
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Inserting DNA into bacteria and making gene libraries.

SESSION 5

D. Micklos and M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory Results: Inserting DNA into bacteria and human DNA fingerprinting.
K. Culver, National Institutes of Health, Bethesda, Maryland: The first human gene therapy trials.

SESSION 6

R. Davis, Cold Spring Harbor Laboratory, New York: Searching for the genetic basis of learning and memory.

J. Forehand, V. Logan, M. Bloom

DNA Forensic Fingerprinting

December 13–December 15

FUNDED IN PART BY

New York State Division of Criminal Justice Services

ARRANGED BY

J. Ballantyne, Suffolk County Crime Laboratory, Hauppauge, New York
T. Marr, Cold Spring Harbor Laboratory, New York
P.F. Palmedo, Long Island Research Institute, Setauket, New York

SESSION 1: Overview of Policies and Strategies

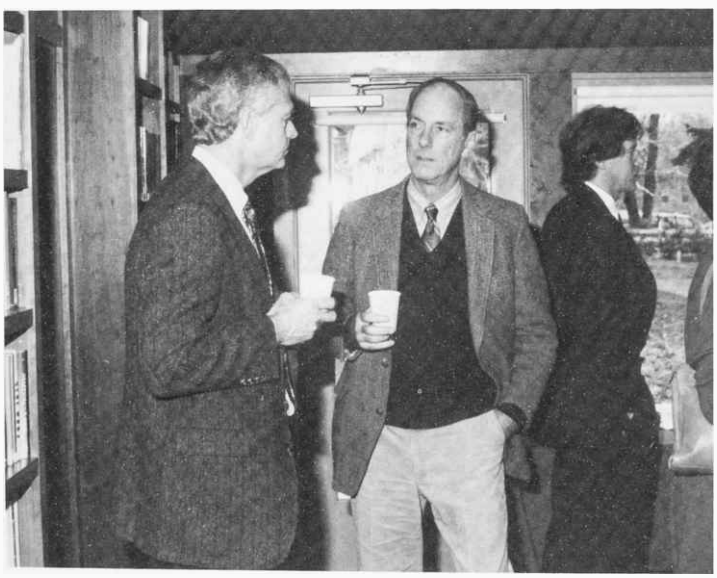
Chairperson: V. Crispino, Suffolk County Crime Laboratory, Hauppauge, New York

J.W. Hicks, Federal Bureau of Investigation Laboratory, Washington, D.C.: Federal and national systems.
R. Girgenti, New York State Division of Criminal Justice Services, Albany: New York State.

SESSION 2: Review of Current Systems

Chairperson: V. Crispino, Suffolk County Crime Laboratory, Hauppauge, New York

J.R. Brown, Federal Bureau of Investigation Laboratory, Washington, D.C.: The FBI system.
J.D. Ban, Virginia Division of Forensic Sciences, Richmond, Virginia: The State of Virginia.
D. Coffman, Florida Department of Law Enforcement, Tallahassee: The State of Florida.
F.C. Dolejsi, Minnesota Department of Public Safety, St. Paul: The State of Minnesota.



J. Hicks, P.F. Palmedo

SESSION 3: Review of New York State Laboratories

Chairperson: R. Girgenti, New York State Division of Criminal Justice Services, Albany

R. Horn, New York State Police Crime Laboratory, Albany
B. Duceman, New York State Police Crime Laboratory, Albany
J. Ballantyne, Suffolk County Crime Laboratory, Hauppauge, New York
J.P. Simich, Erie County Central Police Services, Buffalo, New York
S. Wanlass, Nassau County Police Department, Mineola, New York
L. Duffy, Westchester County Department of Laboratories and Research, Valhalla, New York

SESSION 4: Developments in Computer Databases

Chairperson: K.L. Monson, FBI Academy, Quantico, Virginia

T.G. Marr, Cold Spring Harbor Laboratory, New York: New database technologies.
M. Cinkosky, Los Alamos National Laboratory, New Mexico: Ten year's experience with running an international genetics database.
S.T. Smith, Los Alamos National Laboratory, New Mexico: Comments on computer security.

SESSION 5: Presentation and Discussion of Pilot Project

Discussion Leader: T.G. Marr, Cold Spring Harbor Laboratory, New York

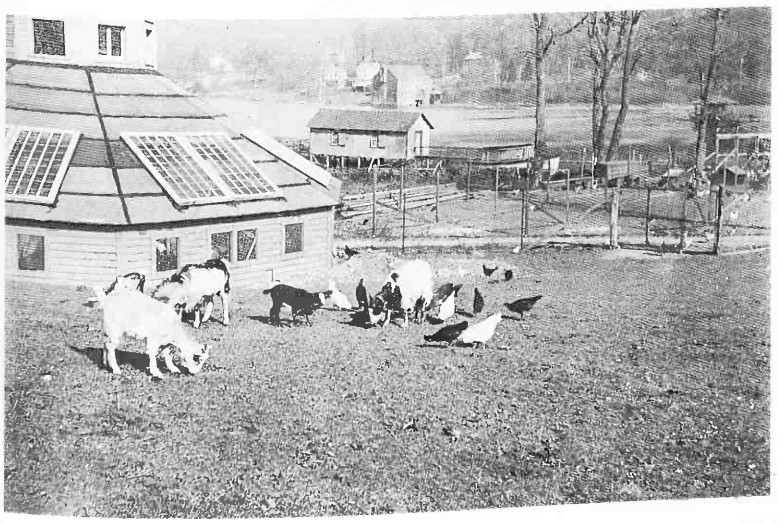
T. Marr, K. Monson

DNA LEARNING CENTER

David A. Micklos, Director
Mark V. Bloom, Assistant Director
Susan M. Lauter, Designer
Jane P. Conigliaro, Education Manager
Robert Willis, Special Programs Manager
Sandra H. Ordway, Administrative Assistant

During his tenure as founding director of the National Center for Human Genome Research, Laboratory Director James Watson called for at least 3% of genome research funds to be devoted to examining the ethical, legal, and social implications of genetics research. This was a bold and unprecedented step to tie biological research directly to its social effects. Dr. Watson's sensitivity to the social issues of genetics was heightened by his knowledge of its history. Indeed, the New York metropolitan area provides an especially good vantage point from which to examine the striking parallel between the rapid advance of molecular genetics and its applications to human disease in the last several decades of the 20th century, and the explosive birth of modern genetics and its applications to eugenics during the first several decades of the century.

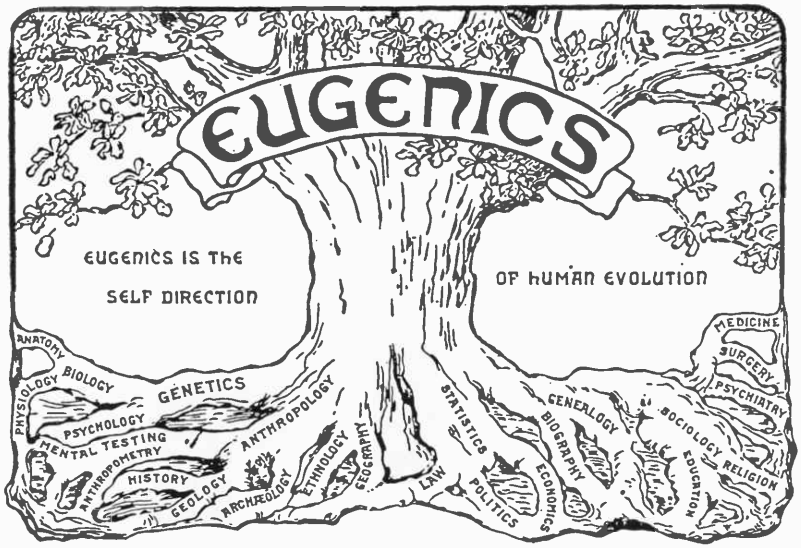
The era of modern genetics began in 1900 with the rediscovery of Mendel's laws, which had laid dormant in an obscure European journal since 1865. Researchers who were then trying to recreate evolutionary processes under controlled conditions—the so-called experimental evolutionists—were quick to adopt Mendelian genetics as a tool for analyzing inheritance in their laboratory plants and animals. From such a background came Thomas Hunt Morgan, whose group at Columbia University was largely responsible for elaborating the mechanisms of Mendelian inheritance, and Charles Davenport, director of the Station for Experimental Evolution at Cold Spring Harbor (the forerunner of the current Laboratory) from 1904 to 1924. Davenport and other early practitioners of genetics also had backgrounds in plant and animal breeding. Thus, the Station for Experimental Evolution resembled an experimental farm, complete with cornfields, hen houses, and grazing sheep.



The Station for Experimental Evolution

genetics to human inheritance. Davenport helped usher in the study of human genetics with a 1907 publication in the journal *Science* explaining the inheritance of blue and brown eye color, and he later published papers on epilepsy, Huntington's chorea, albinism, and neurofibromatosis. Mendelian analysis of human characteristics merged easily with the growing eugenics movement, founded in Europe in the late 1800s, which Davenport aptly described as "the science of human improvement through better breeding." In 1910, Davenport obtained sponsorship from the widow of E.H. Harriman (the railroad robber baron) to establish a Eugenics Record Office at Cold Spring Harbor. Until its disbandment in 1940, the Eugenics Record Office was the epicenter of the American eugenics movement, training eugenics field workers to collect pedigree data and amassing 750,000 genetic records. The institution also provided advice "concerning the eugenical fitness of proposed marriages"; eugenic family histories were fashionable among families who could afford the cost. By 1920, Eugenics exhibitions and "Fitter Families Contests," appraising the genetic quality of human families, were found alongside livestock and produce competitions at state fairs.

The eugenicists were not content merely to chart human inheritance or haggle over whose families were fittest, they began to develop very definite ideas about what constituted the right genetic stuff. Equipped with "scientific" studies purporting to show the Mendelian inheritance of socially unacceptable traits such as nomadism, feeblemindedness, violent temper, lack of moral control, and shiftlessness, they began to force through restrictive and coercive eugenics legislation. Eugenicists' "data" on the genetic inferiority of Jews, blacks, Asians, and southern and eastern European immigrants provided the "scientific" basis for the Johnson Immigration Restriction Act of 1924, which effectively closed the floodgate on the waves of early 20th century immigrants who arrived primarily at Ellis Island, New York, and from whom a majority of Americans living today are descended. By 1931, 30 American states had laws allowing the involuntary sterilization of sex offenders and habitual criminals, as well as the insane and mentally retarded. Historians now believe these mandates were also used as an excuse to sterilize individuals on the basis of race and socioeconomic status. Germany passed its first law for the compulsory sterilization of "hereditary defectives" in



A portion of a certificate awarded to meritorious exhibits at the Second International Congress of Eugenics in 1921 at the American Museum of Natural History.

1933; children of mixed race were sterilized beginning in 1937; euthanasia of children with birth defects commenced in 1938; and "special actions" to exterminate Jews, Gypsies, and other "undesirable elements" began in 1941.

It is easy to dismiss the eugenics movement as pure quackery that could never again be foisted upon society. However, eugenics was branded only in retrospect. In its time, the eugenics movement was respectfully regarded as bona fide science and drew into its circle a number of eminent scientists, philanthropists, and institutions. The recent "ethnic cleansings" in Cambodia and the former Yugoslav Republics illustrate the ever-present potential for abuse of personal freedom in a social climate that values racial purity.

The Social Consequences of Genetic Screening

Perhaps the most immediate social challenge presented by the Human Genome Project will come from the rapid growth of mass genetic screening to detect asymptomatic individuals at risk of developing a disease or of passing a disease gene on to offspring. DNA-based tests are now available for approximately 40 genetic diseases, but the current expense of such tests limits screening primarily to individual families with known risk factors. As technology becomes less expensive, population genetic screening should become as commonplace as cholesterol screening is today. Nobel laureate Walter Gilbert, who discovered a key method for sequencing genes, predicts that *routine* screening will be available for as many as 50 genetic conditions by the year 2000 and for several thousand diseases by 2010.

Retrospective studies of the first mass-screening programs of the 1970s—notably for Tay-Sachs disease and sickle cell anemia, as well as contemporary programs for screening neural tube defects and X-linked disorders—confirm several dangers to be faced as genetic screening becomes widely available:

- Acceptance of genetic screening correlates with increased income and formal education.
- Understanding and retention of genetics information presented by medical professionals correlates with increased income and formal education.
- Families with greater increased income and formal education are less willing to accept the risk of bearing a disabled child.
- Misunderstanding and misinformation about carrier status can stigmatize asymptomatic carriers as being at increased risk for medical problems and subject them to employment and insurance discrimination, as well as psychological distress.
- Many women who receive a positive screening result indicating the possibility of a serious genetic defect in their unborn fetus show little understanding of the testing process, the seriousness of the situation, or their options. These women regard the discovery of an unexpected genetic defect as a medical emergency, whose resolution should be decided by their doctor, rather than as a potential ethical dilemma for themselves.

These results are consistent with National Science Foundation studies conducted over the last three decades, showing that scientific literacy and attention to scientific information correlate with increased income and formal education. In

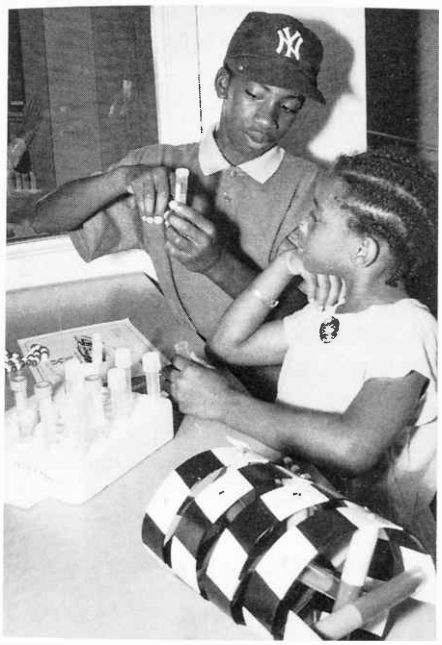
conglomerate, the data suggest that a clinic or doctor's office is not the proper context for a first exposure to genetic testing and that public education campaigns and counseling primarily affect those individuals with some pre-exposure to genetics concepts. Poorer and less educated individuals are more likely to forego genetic testing or cede decisions about genetic testing to their doctors. This suggests a frightening scenario in which poorer and less educated families face an increasing burden of genetic disease, driving them further into disadvantage.

Funding from the Ethical, Legal, and Social Issues Program of the Human Genome Project is currently focused on informed patient consent to genetic testing and protection of private genetic records. Many argue that these issues have, in fact, already been thoroughly examined in similar contexts of general medicine. However, relatively little funding has been devoted to the problem of building a genetically literate public that understands elements of personal genetic health and participates effectively in policy issues of genetic information. Even the most informed of our great private foundations involved in education and social policy still regard genetics as an essentially scientific problem. They have yet to internalize the looming social impacts—both hopeful and distressing—of genetic medicine at the close of the 20th century.

Extending our Expertise to Minority and Disadvantaged Settings

If we accept the premise that minority populations stand the greatest risk of being excluded from the benefits and harmed by the misuse of genetic technology, then genetic literacy issues are especially relevant to the metropolitan New York area, an historical melting pot of minority, racial, and ethnic groups. Minorities compose 64% of 1.3 million precollege students enrolled in public schools in New York City, Nassau County, and Suffolk County. Many of these children are ill-prepared by home experiences when they enter elementary school and are at elevated risk of educational failure. Stigmatized as slow learners, at-risk students are often given remedial exercises that do little to develop problem-solving skills or to place learning in the context of their own interests and experiences. Thus, they fall further and further behind throughout elementary and secondary school. This vicious cycle appears to affect science interest and aptitude disproportionately; the percentage of minorities receiving undergraduate and graduate degrees in scientific disciplines is far below their representation in the general population.

We believe that genetics is an especially appropriate vehicle to reconnect minority/disadvantaged students with science. As a paradigm of "whole learning," genetics offers an almost unparalleled opportunity to integrate concepts across disciplines and to relate technology to an individual's life and culture. Genetics issues entail discussions of personal autonomy, allocation of public resources, and equity for women, minorities, and the disabled. Emphasizing sensitivity to minority and disabled issues of genetics is consistent with the objectives of the Americans with Disabilities Act and New York City's so-called "rainbow curriculum." The DNA Learning Center's (DNALC) minority/disadvantaged outreach is an interdisciplinary micro-application of the principles embodied in the Accelerated Schools Project of Stanford University and the Center for Educational Innovation of the Manhattan Institute. These and other projects have "rescued" urban schools from the downward spiral into academic failure by emphasizing academic acceleration, teacher innovation, and community involvement.



A participant in a *Fun With DNA* summer camp for minority and disadvantaged students explains his DNA extraction experiment to his younger sister.

The recruitment of minority educator Robert Willis, in July, allowed us to initiate a measured, substantive response to the problems of minority/disadvantaged genetics education. With support from the William Randolph Hearst Foundation and the Barker Welfare Foundation, three *Fun With DNA* summer camps were held in August serving 54 minority and disadvantaged students from ten Long Island schools. Contributions from Long Island businesses allowed us to provide scholarships for 155 students to attend laboratory field trips to the *Bio2000* Laboratory during the academic year.

Advance planning was completed on an *Intensive Enrichment* Program, which will become our major vehicle to provide in-school and supplemental resources to support the science literacy needs and career aspirations of minority and disadvantaged students in the New York metropolitan area. The object is to use genetics as a focal point for accelerated science instruction extending from upper elementary through high school. Five *Resource Clusters* of elementary and middle schools will be built around hub high schools in Harlem, Brooklyn, Queens, the Bronx, and mid-Long Island. Each of the five hub high schools will maintain basic equipment sets needed for genetics laboratories to be shared with elementary and middle schools in their *Resource Cluster.* Specialized equipment will be available on-loan from the DNALC for advanced experiments and student research projects. Faculty will receive training during the summer through the DNALC's established workshops. Modeling of teaching strategies by DNALC staff and easy availability of technical support will lead *Intensive Enrichment* teachers into independent instruction.

Wealthy school districts of Long Island have been the proving ground and initial beneficiaries of the DNALC's curricula and teacher training. Thus, we also envision a "Robin Hood" aspect to the *Intensive Enrichment* program through which these wealthy school systems can work together with resource-poor school systems to promote excellence in science education. A number of Long Island school districts with well-established genetics instruction have agreed to be part-

nered with *Intensive Enrichment* schools for classroom observation, teacher mentoring, and cooperative student projects. Previously trained faculty in the partner districts will be linked in a resource group with *Intensive Enrichment* faculty to stimulate innovation, curriculum coordination, and resource sharing across grade levels.

We also want to help establish the *Intensive Enrichment* schools as "community science centers" to provide continuing genetics education to parents and community members. By stimulating collaboration between teachers, parents, community service groups, genetics researchers, genetic counselors, and genetic disease foundations/support groups, we hope to develop community infrastructures to support diffusion of public health messages. We intend to rotate to each *Resource Cluster* our mobile mini-exhibit on genetics—the *Genetic Video Arcade*. The arrival of the exhibit will create a significant "event" around which to organize a rich program of in-school field trips and seminars, student laboratories, and a community genetics fair. Analogous to a student science fair, the community genetics fair would include a number of public events, including seminars, panel discussions, dramatic presentations, debates, mock trials, student laboratory demonstrations, and research projects.

An Educational Imperative

Modern constructivist and cognitive models of learning assert that individuals build knowledge by integrating new information into a pre-existing network of associations. According to these models, decisions about personal and social impacts of genetics must be tied to pre-existing knowledge and value systems developed during childhood and adolescence. This line of reasoning leads to the inescapable conclusion that the precollege school system must be responsible for ensuring that all school children receive sufficient pre-exposure to genetics to empower their future decision-making. This conclusion has been echoed at least twice in the last two decades in the strong recommendations of a 1975 National Academy of Sciences panel and a 1983 Presidential Commission:

> It is essential to begin the study of human biology, including genetics and probability, in primary school, continuing with a more health-related program in secondary school.... Sufficient knowledge of genetics, probability, and medicine leading to appropriate perceptions of susceptibility to and seriousness of genetic disease and of carrier status cannot be acquired as a consequence of incidental, accidental, or haphazard learning.
>
> Efforts to develop genetics curricula [at all levels] and to work with educators to incorporate appropriate materials into the classroom...should be furthered. The knowledge imparted is not only important in itself but also promotes values of personal autonomy and informed public participation.

There is growing awareness among educators that a basic understanding of genetics, disease risk, and health choices is an essential element of cultural literacy—as important in the education of a developing child as is a basic understanding of hygiene and nutrition. Systematic genetics education should begin in elementary school with principles of human variability, inheritance, and disease risk; progress in middle school to more formal aspects of Mendelian analysis, modern gene manipulation, and genetic testing; and culminate in high school

with elements of gene regulation and signal transduction. This effort would fulfill three important objectives, which essentially parallel student development:

- To inculcate basic tenets of genetic literacy that are essential for all students as they assume management of personal and family health care as adults.
- To help prepare college-bound students for their future roles as opinion leaders in government, industry, education, medicine, and law.
- To maintain and broaden the interest of the approximately 15% of science-interested students who are intent on biology or health-related majors, and to stimulate this interest in other motivated students.

Teacher Training and Evidence of Success

The social imperative of the Human Genome Project demands the development of teaching resources capable of bringing science education into the gene age. However, the majority of precollege teachers in the United States have been out of college for 10–20 years, precisely the period of explosion in genetic biology. The majority of all 10th to 12th grade science teachers have completed a Master's degree, generally needed for salary advancement and tenure, so fewer are returning to college for continuing education. At the same time, evidence suggests that most teachers rely heavily on textbooks, which tend to emphasize the formal aspects of Mendelian inheritance using plant and animal examples.

With these problems in mind—and in advance of the Human Genome Project—the first major precollege training programs in molecular genetics were initiated in 1985, notably here and at Georgetown University. Our analysis of a genetics education database developed by the National Academy of Sciences indicates that there are now approximately 30 major, ongoing training programs for precollege teachers in genetics/biotechnology that are administered through academic institutions in the United States. The majority of these programs focus on molecular genetic techniques, are targeted at the high school level, and are clustered in the urban east, California, and several midwestern states. Most training programs include the "core" techniques of transformation (putting DNA into bacteria), restriction (cutting DNA with enzymes), and ligation (recombining DNA). Student laboratories on these core techniques were not taught prior to 1985, and gearing up for them entails significant teacher investment in supplies, equipment, and time.

The rapid adoption of these relatively sophisticated laboratories in molecular genetics at the advanced high school level provides a clear example of the dramatic effects of teacher training, especially when coupled with a national mandate for innovative instruction. Follow-up surveys of 443 faculty trained by DNALC staff in 1987–1988 showed that significant numbers had implemented laboratories on transformation (35%), restriction (25%), and ligation (18%) during the school year following their workshop experience. The notion that hands-on experience in molecular genetics is essential for college-bound students was legitimized in 1989, when the Educational Testing Service recommended two of the core DNA manipulation laboratories for students who take the nationally administered Advanced Placement Biology curriculum. Following this "mandate," implementation of the core laboratories rose substantially for 242 teachers trained in 1989–1990: 51% for transformation, 42% for restriction, and 27% for

A *Fun With DNA* participant examines fruit fly mutations with a family member on the final Parent Participation Day of the workshop.

ligation. Extrapolating these rates to the estimated 3,500 American teachers trained thus far suggests that there are approximately 100,000 precollege student exposures to core DNA manipulation laboratories annually, and $2 million are spent on needed equipment, supplies, and reagents.

Analysis of our database also suggests that several hundred American high schools now include substantial laboratory-based units or elective courses in molecular genetics. Laboratory genetics units are typically incorporated into existing electives, such as anatomy, physiology, biology II, honors biology, and research. In collaboration with Fred Gillam, we completed a survey of 190 college students who had taken the molecular genetics course he has offered at Sachem High School since 1987. The data strongly suggest that genetics electives, like that offered at Sachem, influence science-interested students to choose college majors in biology and health-related disciplines and to consider careers in these fields. The data also suggest that genetics courses preferentially support young women's aspirations for careers in the biological sciences.

Moving Genetics Education into the Elementary Schools

Despite evidence of success of introducing modern genetics at the advanced high school level, we and others have come to regard the elementary schools as the first front in the battle for genetic literacy. Here, student and teacher enthusiasm, flexible scheduling, and lack of standardized curricula greatly simplify instructional change. Elementary teachers, who work with the same class of children all day, have an unparalleled opportunity to use genetics as the hub of a rich learning network that links skills and concepts across the disciplines of reading, writing, math, and science. This is consistent with a recommendation of the National Research Council's 1990 study of American biology education that "science stories" be integrated into elementary language arts. Early genetics education should focus on human development, variability, and health. Sensitive dialogue on social and ethical issues may be especially important to elementary children, who are in the process of forming fundamental attitudes. Genetics also offers ideal subject matter to explore elements of probability included in the national math standards for students, including collecting and analyzing data, drawing samples, and comparing predicted versus experimental ratios.

The DNALC's initial forays into elementary genetics education date to 1989 when we piloted the *Fun With DNA* summer camp. With funding from the William Randolph Hearst Foundation, in 1991 we expanded the summer program to six camps and initiated an academic-year program of laboratory field trips for elementary and middle school students. This year, we began a major new initiative, *Genetics as a Model for Whole Learning in Elementary Science*. Six local school districts—Commack, Great Neck, Half Hollow Hills, Locust Valley, Plainedge, and Roslyn—each pledged $10,000 over two years to support the systematic introduction of genetics modules in their elementary and middle school classes. The object is to train classroom teachers, usually with backgrounds in reading and language arts, to use genetics as a means to link science with the more familiar disciplines of math, geography, and the humanities. Under the program, each district receives 100 hours of consultation with DNALC Education Manager Jane Conigliaro. The program's first year involved 27 faculty and 1,000 students at 15 elementary and middle schools.

In the small school districts of Long Island, we are thus beginning to see working models of science education for the gene age, incorporating hands-on learning about genetics at several stages during child and adolescent development. Typically composed of only two or three elementary schools, one middle school, and one high school, these districts present ideal situations in which to introduce coordinated and system-wide instructional innovation. This is exactly what we envisioned in 1985, when we began our educational efforts on Long Island.

Another Busy Year of Laboratories, Lectures, and Workshops

The *Bio2000* Laboratory was kept very busy during the academic year, with 3300 precollege students participating in laboratory field trips. During a typical week, we now offer five high school laboratories in the mornings and three middle school laboratories in the afternoons. Teachers choose from a "menu" of laboratories, designed to complement science courses typically offered at local schools:

	Grade Level	Time
Variability and Inheritance	5–8	1.5 hours
Corn Genetics and Mendelian Inheritance	5–8	1.5 hours
Cells, Chromosomes, and Mutations	5–8	1.5 hours
DNA Structure and Recombination	5–8	1.5 hours
Bacterial Transformation	9–12	2 hours
DNA Restriction Analysis	9–12	3 hours
Human DNA Fingerprinting	11–12	2 hours

The *Great Moments in DNA Science* Lecture Series, held in the spring, continued as a popular element of our annual calendar of events, drawing the attendance of 1,000 students and teachers. Included in the audience were many "regulars"—faculty who have attended each year since the inception of the lecture series in 1985. This year's lectures aptly illustrated molecular approaches to basic biological problems, as well as applications in health and law:

"The First Human Gene Therapy Trials," Kenneth Culver, National Institutes of Health.

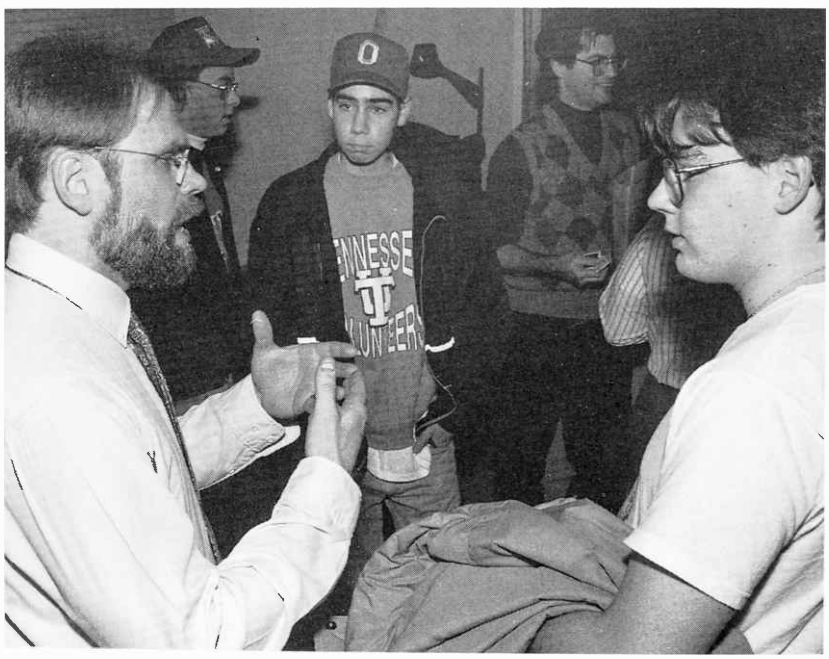
"Using DNA in Criminal Investigations," Robert Shaler, New York City Medical Examiner's Office.

"The Molecular Basis of Learning and Memory," Ronald Davis, Cold Spring Harbor Laboratory.

"Human Immunodeficiency Virus Variability," Winship Herr, Cold Spring Harbor Laboratory.

Summer has traditionally been our busiest time, and the summer of 1992 was no exception. With support from the National Science Foundation, the Department of Education, and the Howard Hughes Medical Institute, a total of 18 training workshops were conducted for elementary through college faculty. Margaret Henderson assisted lead middle school teachers trained in previous summers to conduct "second-round" workshops for 80 additional faculty in New York and Maryland. The popular *DNA Science* Workshop—taught by David Micklos and now in its eighth year of operation—reached 155 high school teachers at workshops held in Arkansas, Kentucky, Maryland, Nevada, and Puerto Rico. Mark Bloom presented his *Advanced DNA Science* Workshop to 100 college faculty at workshops in New York, Washington, D.C., Illinois, and Puerto Rico. Student workshops are becoming an increasingly large component of our summer activities. The DNALC was site for six *Fun With DNA* summer camps attended by 128 5th and 6th graders, including three workshops especially designated for minority and disadvantaged students. Research-oriented high school students (19) from local school districts outnumbered teachers at the *DNA Science* Workshop held at the DNALC.

With funding from the Department of Energy's program on the Ethical, Legal, and Social Issues of Human Genome Research, we continued to hold workshops for opinion leaders and public policy makers. Each of two workshops held in 1992 drew together an eclectic group of administrators and communicators from federal and state governments, genetic support groups, foundations, associations, the mass media, the legal community, and the ethics community—all of



Kenneth Culver addresses student questions after his *Great Moments* lecture on gene therapy.

James Watson (second from right) speaks with *Advanced DNA Science* follow-up workshop participants after his talk on the Human Genome Project.

Cindy Cutshall (second from left) performs a laboratory with her parents Susan (at left) and William Cutshall during the DOE opinion leaders workshop. Cindy is one of the first individuals undergoing gene therapy for ADA deficiency.

whom desire to deepen their insight into human genetics. Most participants come to the workshop with rather extensive "book knowledge" on genetics, but with only fragmentary understanding of the technology upon which modern genetic analysis is based. Thus, the workshop aims to deepen insight into the research process and to fill in gaps in participants' understanding. Jointly administered with our friend Jan Witkowski, the 3-day workshop combines high-level seminars at his Banbury Center with hands-on laboratories at the DNALC that illustrate elements of chromosome mapping, gene library construction, and human DNA polymorphisms. Seminars provided experts' views of the progress and problems of genetics research:

"The Human Genome Project," James Watson, Cold Spring Harbor Laboratory.

"Population Screening for Genetic Diseases," Nancy Press, UCLA Medical Center.

"Counseling for Human Genetics Diseases," Patricia Ward, Baylor College of Medicine.

"Ethical Implications of Human Molecular Genetics," Thomas Murray, Case Western Reserve University School of Medicine.

"Analyzing the Molecular Genetics of Cancer," Eric Fearon, Johns Hopkins University School of Medicine.

"Searching for the Genetic Basis of Learning and Memory," Ronald Davis, Cold Spring Harbor Laboratory.

"The First Human Gene Therapy Trials," Kenneth Culver, National Institutes of Health.

Initiating a Major Capital Development Program

The Laboratory's Board of Trustees has endorsed a $3.5 million capital development program to create the physical resources for the DNALC to take on an expanded role as a prototype "Human Genome Education Center." The three-phase program is expected to be completed in 1994:

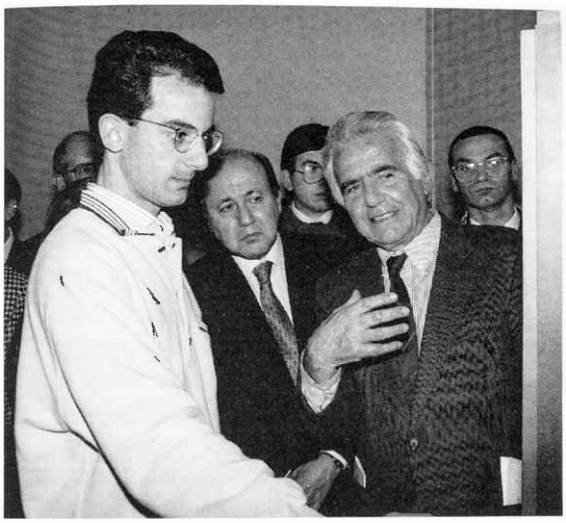
- Phase I: Purchase of the DNALC property from Cold Spring Harbor Central School District at the conclusion of its lease/option on December 31, 1992.
- Phase II (January–June, 1993): Redevelopment of the existing property, including renovation of a 104-seat auditorium, renovation of galleries, installation of new exhibits, installation of a computer multimedia laboratory, installation of fire sprinklers, and improvement of visitor parking and handicap access.
- Phase III (Fall 1993–Fall 1994): Construction of a 3,500-square-foot *BioMedia* addition to the south side of the building, including a second teaching laboratory, library, and an atrium/lunchroom. The term *BioMedia* engenders our goal to explore ways to link experimental, computer, and audiovisual resources to encourage understanding of biological concepts. The new and redeveloped facilities will allow students to move between biochemical experiments, microscope observations, and parallel computer experiences that illustrate molecular events.

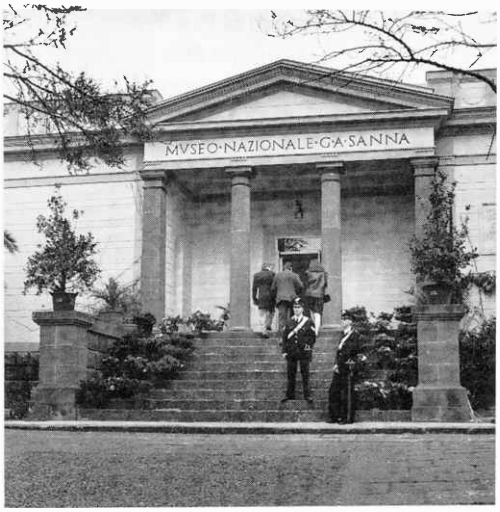
The capital program is based on an architectural design by Centerbrook Associates, who have been responsible for architectural design at the Laboratory for more than a decade. In November, the Suffolk County Industrial Development Agency approved a tax-exempt municipal bond for the purchase and redevelopment of the facility. Approval to proceed with the capital program was based upon receipt of lead grants in 1991 from the Stone Foundation ($250,000) and the Weezie Foundation ($100,000), and in 1992 from Cablevision Systems Corporation ($250,000) and the E.S. Webster Foundation ($50,000). We were also very pleased to receive from Beckman Instruments gifts of an L8-70M ultracentrifuge and GS-6 tabletop centrifuge.

International Collaborations

We were happy for an opportunity to aid long-time friend Marcello Siniscalco in his efforts to bring into operation a new genetics institute on the Sardinian coast: the Porto Conte Research and Training Laboratories. In the context of recent revelations in Italy of millions of dollars misspent for bogus public works projects, the Porto Conte Laboratory is one of only a very few successful projects to bring technological development to the poorer regions of Italy. Sardinia is an almost ideal site for a laboratory on human genetics. The Sardinian people represent essentially a closed gene pool, derived from a distinctive prehistoric culture and cut off from major immigration for centuries.

Marcello also has received seed money from the Italian Ministry of University, Scientific, and Technological Research, to develop a plan for an education center adjacent to the research laboratories. To help publicize this educational mission, we were subcontracted to develop a prototype *Genetic Video Arcade* for display in Sardinia. Susan Lauter collaborated with Paola Melis to translate four





Marcello Siniscalco of Sardinia, Italy views one of the computer presentations of the *Genetic Video Arcade* displayed at the Museo Nazionale in Sassari (right).

interactive computer presentations into Italian and to mail 1,000 pounds of computers and display modules to Sardinia. Four computer modules and a wide-screen video projector were installed in the Museo Nazionale Sanna in Sassari. The *Genetic Video Arcade's* frank modernism—in design and content—contrasted with a contemporaneous exhibit of Columbian documents and the museum's noteworthy collections of bronze-age artifacts. The arcade was visited by 7,000 students during the Italian national "Week of Scientific Culture," April 8–16, which included lectures by Italian Nobel laureate Luigi Cavalli-Sforza and Sir Walter Bodmer, director of the Imperial Cancer Research Fund. Response in Sassari was so favorable that the *Genetic Video Arcade* was set up in Genoa as part of the Columbian 500th anniversary celebrations in June.

We initiated a new collaboration with the municipality of Svalov, a small agrarian community in Skania Province of southern Sweden. Only a brief ferry ride away from Copenhagen, Skania has a long history in agricultural genetics. The horse-breeding station at Flyinge has functioned continuously since the 12th century and has been responsible for developing the Swedish "warm-blood," which is in great demand for high-level equestrian sports. The company Svalof AB was established in 1886 to develop grain, forage, vegetable, and oil seeds for use by farmers throughout Sweden. Thus, it is not surprising that the municipality of Svalov is now developing plans for a science education center to focus on agricultural genetics.

After visiting the DNALC in January, the Svalov planners decided that the DNALC was a useful model for the science center they envision in Southern Sweden. Four elementary and high school teachers from Svalov were trained to perform hands-on genetics laboratories during summer workshops at the DNALC. To help publicize the proposed science center, David Micklos was invited to participate in "Gene Vision," an exhibit on agricultural genetics and accompanying interpretive activities during several weeks in October. The exhibit included Swedish translations of two computer presentations from our *Genetic Video Arcade*; seminar speakers included Swedish scientist Ulf Pettersson, who did postdoctoral research at Cold Spring Harbor Laboratory in 1971–1972. The exhibit received 1500 visitors, and 1200 persons (aged 9–65) participated in hands-on laboratories on DNA restriction, bacterial transformation, DNA structure, and Mendelian genetics.

In early summer, Margaret Henderson left the DNALC to accept a position as Head of Library Services at the main Laboratory campus. As Education Manager, Margaret fit in easily with the rest of our small staff and proved herself as a gifted instructor and untiring organizer. We were happy that Margaret was able to find within the Laboratory a position that so well utilizes her graduate training in library science, but we miss her team spirit and even disposition.

The loss of Margaret from our staff was eased by our success in recruiting Robert Willis, who joined the DNALC staff in July. As Special Programs Manager, Robert is responsible for developing and administering programs targeted for minority and economically disadvantaged students in the New York metropolitan area. A native of Tallahassee, Florida, Robert received his undergraduate degree in biology from Florida State University. Prior to joining the DNALC, he taught biology, general science, chemistry, and math at Ballou Senior High School in Washington, D.C. He has also taught English as a Second Language to non-English speaking adults and served as an extension math and science teacher. In recent years, Robert received teacher fellowships from the American Society for Biochemistry and Molecular Biology, the Cafritz Foundation, and the Foundation for Advanced Education in the Sciences. As part of his fellowship research, he conducted a study of biotechnology curricula and developed plans to integrate biotechnology into the curriculum of the Washington, DC public schools; he intends to continue this work while at Cold Spring Harbor.

We were also thankful for the opportunity to work with Twana Adams, another gifted and energetic minority educator. An instructor at the Bronx Comprehensive Night High School, Twana assisted with the minority *Fun With DNA* camps and organized a very successful training workshop that drew minority educators from throughout the New York metropolitan area. We anticipate that Twana will play a key role in organizing our new *Intensive Enrichment* program, acting as our agent in her hometown Harlem, as well as in the Bronx.

The laboratory-intensive nature of the *Fun With DNA* summer camps requires increased supervision for 5th and 6th grade participants. Assisting Jane Conigliaro with laboratory instruction at the 1992 camps were Diane Jedlicka, of the Roslyn OMNI Gifted Program, and Adele Nicefero, of the Hicksville Academic Enrichment Program. We owe a special debt of gratitude to these lead educators, and their students, for bringing genetics education into the elementary classroom. They were among the very first to use the DNALC as a resource for

Twana Adams (above, at right) and Robert Willis (below, second from right) oversee *Fun With DNA* students.

DNA Learning Center Staff. (*Top*) Robert Willis, Mark Bloom, Sandy Ordway, Mark Staudinger, David Hollman. (*Bottom*) Jane Conigliaro, David Micklos, Susan Lauter, Ken Bassett.

gifted/talented instruction and provided the impetus for the initial development of the *Fun With DNA* camp. Student aides Tara Marathe, of Northport High School, and Lina Hwang, of SUNY, Stony Brook, provided campers an additional measure of personal attention.

Student interns from neighboring school districts worked behind the scenes to prepare reagents used in student field trips during the academic year and for teacher training workshops during the summer. After ably assisting Mark Bloom with an intense 8-week schedule of college faculty workshops in the summer, Amy Phillips joined the sophomore class at SUNY, Geneseo, to continue her major in biochemistry. Following Amy's departure, Mark Staudinger, of Cold Spring Harbor High School, filled her position as senior intern. Mark assisted with 4 weeks of summer faculty workshops and assumed responsibility for overseeing several new sophomore interns: Ken Bassett, of Massapequa High School; Andrea Conigliaro, of St. Anthony's High School; and Michael Conigliaro, of Cold Spring Harbor High School. Cold Spring Harbor senior David Hollman continued to focus primarily on computer applications, assisting Sue Lauter with the development of multimedia educational programs. We were also fortunate to take on Paul Kwitkin, a senior at Commack High School with background in DNA research at SUNY, Buffalo. Claudio Siniscalco, of Westminster School in London, returned for his second summer, assisting at several faculty workshops and developing multimedia programs.

David Micklos participated in important studies being carried out by two branches of the National Academy of Sciences. He is an appointed member of a National Research Council committee that is assembling a report on effective methods of in-service training for precollege biology teachers. He prepared a background paper that was used as a basis for educational recommendations proposed by an Institute of Medicine committee on genetic testing. Mark Bloom began consulting for Ralph Appelbaum Associates, Inc. on the design of biotechnology exhibits for the new National Science and Technology Museum in Taiwan, due to open in 1993.

Publications

Bloom, M., G. Freyer, and D. Micklos. *Laboratory DNA science: An introduction to recombinant DNA technology and methods of genome analysis.* (In press.)

Micklos, D. Genetic Testing: An educational imperative to our schools. In *Proceedings of the Committee on Assessing Genetic Risks, Institute of Medicine.* (In press.)

Micklos, D. Genetics Education in American Schools: A View From Cold Spring Harbor Laboratory. *Journal of the Swedish Seed Association* **102 (4):** December 1992.

Sites of Major 3- to 10-Day Workshops 1985–1992

ALABAMA	University of Alabama, Tuscaloosa	1987,1988,1989,1990
ARIZONA	Tuba City High School	1988
ARKANSAS	Henderson State University, Arkadelphia	1992
CALIFORNIA	University of California, Davis	1986
	University of California, San Francisco	**1991**
CONNECTICUT	Choate Rosemary Hall, Wallingford	1987
FLORIDA	North Miami Beach Senior High School	1991
	University of Western Florida, Pensacola	1991
	Armwood Senior High School, Tampa	1991
GEORGIA	Fernbank, Inc., Atlanta	1989
	Morehouse College, Atlanta	**1991**
HAWAII	Kamehameha Secondary School, Honolulu	1990
ILLINOIS	Argonne National Laboratory, Chicago	1986, 1987
	University of Chicago	**1992**
INDIANA	Butler University, Indianapolis	1987
IOWA	Drake University, Des Moines	1987
KENTUCKY	Murray State University	1988
	University of Kentucky, Bowling Green	1992
	Western Kentucky University	1992
LOUISIANA	Jefferson Parish Public Schools, Harvey	1990
MANITOBA	Red River Community College, Winnipeg	1989
MARYLAND	Annapolis Senior High School	1989
	McDonogh School, Baltimore	1988
	Montgomery County Public Schools	1990,1991,1992
	St. John's College, Annapolis	**1991**
MASSACHUSETTS	Beverly High School	1986
	Dover-Sherborn High School, Dover	1989
	Randolph High School	1988
	Winsor School, Boston	1987
MICHIGAN	Athens High School, Troy	1989
MISSISSIPPI	Mississippi School for Math & Science, Columbus	1990,1991
MISSOURI	Washington University, St. Louis	1989
NEW HAMPSHIRE	St. Paul's School, Concord	1986,1987
NEVADA	University of Nevada, Reno	1992
NEW YORK	Albany High School	1987
	Bronx High School of Science	1987
	Cold Spring Harbor High School	1985,1987
	DeWitt Middle School, Ithaca	*1991*
	DNA Learning Center	1988(3),1989(2),1990(2), 1991, 1992
	DNA Learning Center	**1990, 1992**
	DNA Learning Center	*1990,1991,1992*
	Fostertown School, Newburgh	*1991*
	Huntington High School	1986
	Irvington High School	1986
	Junior High School 263, Brooklyn	*1991*
	Lindenhurst Junior High School	*1991*

Key: High School Workshops, **College Workshops**, and *Middle School Workshops*

	Orchard Park School, Orchard Park	*1991*
	Plainview-Old Bethpage Middle School, Plainview	*1991*
	State University of New York, Purchase	1989
	State University of New York, Stony Brook	1987,1988,1989,1990
	Titusville Middle School, Poughkeepsie	*1991*
	Wheatley School, Old Westbury	1985
NORTH CAROLINA	North Carolina School of Science, Durham	1987
OHIO	Case Western Reserve University, Cleveland	1990
	Cleveland Clinic	1987
	North Westerville High School	1990
PENNSYLVANIA	Duquesne University, Pittsburgh	1988
	Germantown Academy, Fort Washington	1988
PUERTO RICO	University of Puerto Rico, Mayaguez	1992
	University of Puerto Rico, Mayaguez	**1992**
SOUTH CAROLINA	Medical University of South Carolina, Charleston	1988
	University of South Carolina, Columbia	1988
TEXAS	J.J. Pearce High School, Richardson	1990
	Langham Creek High School, Houston	1991
	Taft High School, San Antonio	1991
VERMONT	University of Vermont, Burlington	1989
VIRGINIA	Jefferson School of Science, Alexandria	1987
	Mathematics and Science Center, Richmond	1990
WASHINGTON, DC	**Howard University**	**1992**
WEST VIRGINIA	Bethany College	1989
WISCONSIN	Marquette University, Milwaukee	1986,1987
	University of Wisconsin, Madison	1988,1989
WYOMING	University of Wyoming, Laramie	1991

1992 Workshops, Meetings, and Collaborations

January 7–8	Site visit by Svalovs Kommun Delegation (Sweden)
January 14	Student Workshop, A. Philip Randolph High School, Harlem, New York
January 16	Grant review, William Patterson College, Wayne, New Jersey
January 18–19	National Science Foundation High School Faculty Workshop, Miami, Florida
January 25	Congressional Aides Workshop
January 25–26	National Science Foundation High School Faculty Workshop, Tampa, Florida
February 2–3	National Research Council Committee Meeting, Washington, DC
February 3–March 3	Site visit by Paola Melis of Porto Conte Research and Training Laboratories, Sardinia, Italy
February 5	Site visit by Jackie Grennon Brooks of SUNY, Stony Brook
February 8–9	National Science Foundation Middle School Faculty Workshop, Annapolis, Maryland
February 15–16	National Science Foundation College Faculty Workshop, San Francisco, California
February 21–23	Seminar, Coalition for Education in the Life Sciences Meeting, Racine, Wisconsin
February 23–26	Department of Energy Opinion Leaders Workshop
February 25	*Great Moments in DNA Science* Student Lecture
February 29–March 1	National Science Foundation College Faculty Workshop
March 11	*Great Moments in DNA Science* Student Lecture
March 15–21	National Research Council Committee Meeting, Irvine, California
March 17	*Great Moments in DNA Science* Student Lecture
March 25	Middle School Faculty Workshop
March 26	*Great Moments in DNA Science* Student Lecture
March 26–28	Workshop, National Association of Biology Teachers Meeting, Boston, Massachusetts
March 30–April 11	"Week of Scientific Culture" Exhibit, Sardinia, Italy
April 1–8	Site visit by Kerry and Bev Clarke, Australia
April 11	Cold Spring Harbor Laboratory Association Workshop
April 11	Congressional Aides Workshop
April 15	Corporate Advisory Board Meeting and Reception
April 21–24	Consultation, Carolina Biological Supply Company, Burlington, North Carolina
April 29	Lecture for Kiwanis of Long Island, Northport
May 6	Grant review, William Patterson College, Wayne, New Jersey
May 6–7	National Science Foundation Middle School Faculty Workshop, Howard County, Maryland
May 7	Lecture, Suffolk County Organization for Promotion of Education
May 27–29	Multi-Media Expo, New York
May 25–June 5	National Science Foundation College Faculty Workshop, University of Puerto Rico, Mayaguez
June 6–7	Middle School Faculty Workshop
June 8-12	National Science Foundation High School Faculty Workshop, University of Puerto Rico, Mayaguez
June 22–July 3	Department of Education College Faculty Workshop, Howard University, Washington, DC
June 22–26	Howard Hughes Medical Institute High School Faculty Workshop, Montgomery County, Maryland
June 23–25	National Science Foundation Middle School Faculty Workshop, Baltimore County, Maryland
June 24–26	National Science Foundation Middle School Faculty Workshop, Frederick County, Maryland

June 29–July 3	National Science Foundation High School Faculty Workshop, Henderson State University, Arkadelphia, Arkansas
June 29–July 3	*Fun With DNA* Student Summer Camp
June 29–July 3	Site visit by Fred Gillam, Sachem High School
July 6–12	*Fun With DNA* Student Summer Camp
July 9–10	High School Faculty Workshop, University of Central Arkansas, Conway
July 13	Grant review, New York Hall of Science, Queens
July 13–17	National Science Foundation High School Workshop, University of Western Kentucky, Bowling Green
July 13–19	*Fun With DNA* Student Summer Camp
July 20–24	National Science Foundation High School Faculty Workshop, University of Kentucky, Lexington
July 21–23	National Science Foundation Middle School Faculty Workshop, Anne Arundel County, Maryland
July 27–August 7	National Science Foundation College Faculty Workshop, Cold Spring Harbor
August 3–7	National Science Foundation High School Faculty Workshop, University of Nevada, Reno
August 3–7	*Fun With DNA* Minority Student Summer Camp
August 10–14	*Fun With DNA* Minority Student Summer Camp
August 10–21	Department of Education College Faculty Workshop, University of Chicago
August 11–13	National Research Council Committee Meeting, Woods Hole, Massachusetts
August 11–13	National Science Foundation Middle School Workshop, Anne Arundel County, Maryland
August 17–21	*Fun With DNA* Minority Student Summer Camp
August 18–20	National Science Foundation Middle School Faculty Workshop, Cecil County, Maryland
August 24–28	High School Student/Faculty Workshop
September 4	National Science Foundation Middle School Faculty Workshop, Ithaca, New York
September 25–27	*Winding Your Way Through DNA* Conference, San Francisco, California
October 12–16	"Gene Vision" Exhibit, Svalov, Sweden
October 16–18	Department of Education Meeting, Washington, DC
October 28–Nov 1	Site visit by Cindy Kelleher, University of California, San Francisco
October 31	Baring Brothers Workshop
November 1	Corporate Advisory Board Presentation and Dinner
November 3	Student Workshop, Valley Stream High School
November 7–8	National Science Foundation High School Faculty Workshop, University of Kentucky, Lexington
November 14	Lecture, Long Island Museum Association Meeting
November 14–15	National Science Foundation High School Faculty Workshop, Western Kentucky University, Bowling Green
November 14–15	Middle School Minority Faculty Workshop
November 19	Workshop, National Association of Science Teachers Meeting, New York
November 21–22	National Science Foundation Faculty Workshop, Henderson State University, Arkadelphia, Arkansas
December 5–8	Department of Energy Opinion Leaders Workshop


COLD SPRING HARBOR
LABORATORY PRESS

1992 PUBLICATIONS

General Books

The Cell Cycle
Symposia on Quantitative Biology 56

DNA on Trial: Genetic Identification and Criminal Justice
P.R. Billings (ed.)

The Dynamic Genome: Barbara McClintock's Ideas in the Century of Genetics
N. Fedoroff and D. Botstein (eds.)

Phage and the Origins of Molecular Biology, expanded edition
J. Cairns, G.S. Stent, and J.D. Watson (eds.)

The Power of Bacterial Genetics: A Literature-based Course
J. Beckwith and T.J. Silhavy

A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli *and Related Bacteria*
J.H. Miller

Vaccines 92: Modern Approaches to New Vaccines Including Prevention of AIDS
F. Brown, R.M. Chanock, H.S. Ginsberg, and R.A. Lerner (eds.)

CSHL Monograph Series

Transcriptional Regulation
S.L. McKnight and K.R. Yamamoto

Current Communications in Cell and Molecular Biology Series

Vol. 5: *Molecular Biology of Free Radical Scavenging Systems*
J.G. Scandalios (ed.)

Vol. 6: *Lyme Disease: Molecular and Immunologic Approaches*
S.E. Schutzer (ed.)

Cancer Surveys Series

Vol. 12: *Tumour Suppressor Genes, the Cell Cycle and Cancer*
A.J. Levine (ed.)

Vol 13: *A New Look at Tumour Immunology*
A.J. McMichael and W.F. Bodmer (eds.)

Vol. 14: *Growth Regulation by Nuclear Hormone Receptors*
M.G. Parker (ed.)

Vol. 15: *Oncogenes in the Development of Leukaemia*
O.N. Witte (ed.)

Genome Analysis Series

Vol. 4: *Strategies for Physical Mapping*
K. Davies and S. Tilghman (eds.)

Journals

Genes & Development (Volume 6, numbers 1–13)

PCR Methods and Applications (Volume 1, numbers 3 and 4; Volume 2, numbers 1 and 2)

Other

CSHL Annual Report 1991

Banbury Annual Report 1991

DNA Learning Center Annual Report

Administration and Financial Annual Report

Abstract/program books for 14 CSHL meetings

COLD SPRING HARBOR LABORATORY PRESS

1992 was a year of unqualified success for Cold Spring Harbor Laboratory Press. Its financial goals were met, with a total income of over $3.7 million, up by over 20% from 1991. The surplus before deduction of allocated Laboratory overhead exceeded $0.51 million, a satisfying result after the loss incurred in 1991. We are still predominantly a book publisher, and book sales contributed more than 60% of our revenues. Journal revenues are increasing annually and for the first time exceeded $1.0 million.

Book Publishing

Within our book program, the most successful titles continue to be our laboratory manuals, monograph series, and the annual Symposium volume. Outstanding additions to each of these categories were made in 1992.

Jeffrey Miller completed his masterly *A Short Course in Bacterial Genetics.* The book combines a manual of techniques refined by constant use in the author's laboratory with a handbook of reference data on *Escherichia coli* and its relatives, organisms with a central place in current biotechnology. The book was eagerly awaited in the genetics community, and we expect it to have the wide appeal and long life span of Miller's *Experiments in Molecular Genetics,* the first of Cold Spring Harbor's laboratory manuals, which was published 20 years ago and is still in print.

The recently revived series of monographs, which began in the early 1970s, continued this year in its classic tradition with a magnificent volume entitled *Transcriptional Regulation,* edited by Steven McKnight and Keith Yamamoto. Conscious of the series' past achievements and undaunted by an enormous and intellectually demanding field, the editors recruited contributions from more than 50 of the leading investigators and worked tirelessly to mold the chapters into a coherent whole. The resultant two-volume set will stand for years at the center of the field's literature.

This year's Symposium volume also maintained a tradition of excellence, focusing on the cell cycle. The book captured the excitement of an area of research in rapid advance, as a growing understanding of the biochemistry of cell division complements awareness of the genetic basis of tumor development in humans.

In total, 15 new book titles were published in 1992. The complete list appears on the opposite page. Each had something special to offer. *The Power of Bacterial Genetics,* for example, provided a novel approach to teaching undergraduates through close analysis of primary research papers, based on the experience of Tom Silhavy at Princeton and Jon Beckwith at Harvard. In *DNA on Trial,* Paul Billings collected a series of essays on the controversial use of DNA typing in criminal justice, to create a book unlike any other currently available.

In its limited space, this report cannot adequately describe all the new book titles in 1992. One, however, must be singled out because of the respect, affection, and secrecy with which it was prepared. *The Dynamic Genome* was born out of a desire, first expressed by James Watson, to surprise Barbara McClintock on her 90th birthday with a book about the science she had influenced so much. From the beginning, it was clear that producing something Barbara herself would

like would be no easy task. To edit it, we turned to Nina Fedoroff and David Botstein, two of her long-time friends and colleagues, whom we knew would assemble the book with taste and care. The 30 invited contributions combined thoughtful analysis of transposable elements in a variety of species with some history, some personal reminiscence, and a discreet amount of eulogy. Published in a striking dust jacket, the book has been widely reviewed and warmly praised. Best of all, it satisfied its subject's own exacting standards. With Barbara McClintock's death in September, the book is now a monument to the legacy of one of the century's great scientists.

In this respect, *The Dynamic Genome* resembles the classic work *Phage and the Origins of Molecular Biology,* published by Cold Spring Harbor in 1966 as a festschrift for Max Delbrück. Hailed as an important document in the history of biology and a formative influence on many of today's leading scientists, the book went out of print in the early 1980s. The editors John Cairns, Gunther Stent, and James Watson agreed with our view that a new generation of molecular biologists deserved a fresh introduction to the book. Cairns wrote a charming memento of Delbrück to preface the new edition, which also included a major review of the original book (and its rejoinder) and Stent's 1982 obituary of Delbrück. The popularity of the reissue has reassured us that despite the overwhelming stream of new research results, molecular biologists remain curious about the roots of their discipline.

The editorial work, design, and production of the new 1992 titles were undertaken with their customary skill and dedication by Managing Editor Nancy Ford and the staff of her department in Urey Cottage. The year's list of new titles was the largest and most complex yet produced by the Press. All but three books were typeset in-house. In the past few years, the book publishing staff have become fewer but have produced more pages. This efficiency has benefited us financially, and the staff's commitment to the highest standards has assured our books' continuing quality and reputation.

Among the backlist titles, the manuals *Molecular Cloning* and *Antibodies* continued to sell strongly, maintaining their dominant position in the technical literature of molecular biology. Sales of the second edition of *Molecular Cloning* now exceed the first. The continued success of two 1991 titles, *Apoptosis* and *Origins of Human Cancer,* was also notable. Both occupy a unique place in the literature of their fields, the first as the only current review of an emergent topic and the second as a comprehensive overview of an enormous and vital area of research.

We continued to distribute large numbers of the remarkable books on biology by Fran Balkwill and Mic Rolph, which originate with HarperCollins U.K. The author/illustrator team achieved strong sales with their latest work *DNA Is Here To Stay,* about the structure of DNA and protein synthesis. Their writing is classified as "children's literature," but it clearly also appeals to adults without a science background who are intrigued by the increasingly public discussion of genetic research. We are delighted with this extension of our publishing activities into more general education and plan to build on this promising beginning.

Marketing Activities

The activities of the Marketing Department were essential to the success of our publishing program in 1992. Conducted practically single-handedly by Ingrid Benirschke, they centered on three subject areas: genetics, cancer biology, and neuroscience. With the creative input of our designer Jim Suddaby, eye-catching

catalogs for each subject were created and mailed to selected customer lists. These were reinforced by three issues of our newsletter *Notebook* and by advertisements in prominent journals that promoted new titles and best-selling backlist books. In the fall, a handsome catalog was widely circulated, containing the complete list of CSHL Press books and journals. The resultant sales response was excellent. Mailings were also done to test reduced-price offers of older titles from our backlist and a special, gift-oriented selection of general interest books and other items. Both experiments were judged successful and encouraged us to pursue these potential new revenue streams.

In addition to its customary reliance on direct mail, the Press exhibited at the annual meeting of six national societies of biological research. These promotional appearances, where our books and journals are available for scrutiny and purchase, are an invaluable and encouraging opportunity to meet and learn from customers in the field.

An ancillary but increasingly important aspect of our marketing activities is the campus bookstore in the Grace Auditorium. Managed by Connie Hallaran, it provides Laboratory staff and meetings attendees with the opportunity to browse among our books and journals and an eclectic selection of science publications from other sources. In 1992, the bookstore's activities expanded, as in the previous year. More books and a greater range of items for visitors were stocked. Despite the small space available, sales increased substantially, encouraging the view that with a greater floor area, the bookstore would have considerable financial potential.

Journal Publishing

Genes & Development ended the year with a consolidated reputation as one of the elite journals in biology. Demand to publish in the journal is growing considerably, and the number of manuscripts submitted rose by more than 13%. In addition, the scope of work available to the journal became broader. The editors, Terri Grodzicker and Nick Hastie, with the help of the advisory board, continued to apply stringent standards of assessment, accepting about 35% of papers offered. The quality of the published work is remarkably high. Although there was only a modest increase in the number of editorial pages published in 1992, the pressure on space in the journal is growing. This is a consequence of the journal's ever-increasing visibility, and discussion is in progress about strategies for dealing with it in 1993 and beyond.

The circulation of *Genes & Development* rose by 4% overall, with increases among both individuals and institutions. The journal has subscribers in over 40 countries, predominantly the United States, Japan, and Western Europe, and circulation increased in each of these markets. The circulation growth, the journal's reputation for editorial excellence, and the caliber of its subscribers offer useful advertising opportunities for product manufacturers. Revenue from this source, however, although considerable, was less than in 1991, owing to economic recession in the commercial sector and growing competition for advertising dollars among scientific journals. New strategies in the approach to potential advertisers are being planned, with the aim of reversing this trend.

The journal's financial performance overall was excellent and met budget projections. Total revenue increased, assisted by circulation growth and a 7–10% increase in subscription prices. Costs were well-controlled and the surplus rose. For CSHL Press, and its minor partner in the journal, the Genetical Society of

tion analysis for 1991 by the Institute for Scientific Information in September showed that the "impact factor" for *Genes & Development* had increased strikingly during 1991 and was again the highest of any monthly journal in biology. By this measure, *Genes & Development* was confirmed as the top journal in genetics and developmental biology.

After the spectacularly successful launch of our new quarterly *PCR Methods and Applications* with two issues in 1991, we were eager to see what would happen to the manuscript submission rate and subscription and advertising sales in the subsequent calendar year. To our great satisfaction, the momentum continued in all three areas. Seventy original papers and six commissioned reviews were published. With the essential help of the associate editors Richard Myers, Eric Green, Richard Gibbs, and David Bentley, editor Judy Cuddihy published papers of a high standard, declining more than half the manuscripts offered for publication. A complimentary review in *Nature* confirmed that the journal was meeting a widespread need for reliable information about the immensely powerful, but technically challenging, polymerase chain reaction. This was underlined by the strong rate of subscription renewal after the first volume in June. We were also gratified by an increase in the number of sponsored subscriptions for Volume 2 purchased by the Perkin-Elmer Corporation. Its support, and the substantial advertising sales solicited by Nancy Kuhle, combined with subscription growth in over 20 countries, created a strong cash flow that enabled the journal, remarkably, to achieve a surplus after only six issues.

The staff of the journal department responded willingly to the challenges presented by more manuscript submissions and larger issues. Led by Managing Editor Judy Cuddihy, they demonstrated an exacting level of care and attention to detail and ensured that all the 1992 issues of both journals appeared on schedule and error-free. They also succeeded in keeping the interval between submission and publication of a manuscript to a minimum. We welcomed Valerie Nicolette to the staff as production editor; she has proved to be a quick and effective study in her first publishing job after graduating from college.

Order Fulfillment and Dispatch

Underpinning any publishing program are vital support services without which editorial and marketing activities would be fruitless. The staff responsible for sales processing, dispatch, and customer service form the front line of communication with purchasers. For many members of the scientific community, this is the sole contact with the Laboratory and its Press. Guy Keyes, the new head of the department, and his colleagues worked hard to improve its efficiency of operation. In 1992, the number of book orders processed and journal subscriptions fulfilled increased substantially, with no increase in staff. More direct mail was dispatched. In addition, the potential of the sales management computer system was exploited more fully, for detailed marketing results, sales data, and customer category analysis.

Financial Management

Further essential support became available in March with the appointment of Nancy Hodson to the new position of Finance Coordinator. With a strong background in finance and accounting, and considerable computer skills, Nancy

quickly impressed us with her quiet efficiency and competence. By the year's end, she had overhauled our accounting and reporting systems, improving staff awareness of financial trends and permitting quicker, more accurate forecasting. With the assistance of financial management software, the flow of information within and from the Press dramatically improved. Controls on spending were tightened, thanks to continuous monitoring of results and budgets. These improvements helped us to achieve our financial targets and gave us the confidence to predict further growth next year.

New Publishing Projects

1992 was forecast as a time of consolidation in which cost-effectiveness could be improved, our book list expanded, and our journal program made securely profitable. These goals were achieved. At the same time, it was necessary to plan for the future, since few publishing projects of any merit can be completed in less than 2 years and many require even longer. During the year, more than 30 new projects of different types were begun, with planned completion dates in 1993–1995. These projects will be described fully in future reports. They include videotapes, which we have found to have a useful place in the communication of science, and potential electronic publications, a medium with extremely interesting prospects. New journal possibilities are also being explored.

But most of these new projects are books. There is no doubt that cash restraints among both libraries and individual investigators have harmed sales of science books, and there is an added tendency among some investigators to believe that the speed of advance in knowledge makes many books out of date as soon as they are printed. However, our market intelligence convinces us that the kind of books Cold Spring Harbor does especially well will continue to be important, even as additional information sources become available. By harnessing new technology and by careful cost control, we have maintained our books' quality at reduced cost, giving them the best chance of financial viability.

A major review of the Press program late in the year gave us a reason to examine the financial history of book publishing at Cold Spring Harbor, from the expansion initiated by James Watson in 1970 to the end of 1991. The income from book sales in this period was close to $25.5 million. After deduction of direct expenses, over $5.0 million has been made available to the Laboratory to support overhead and running costs. We are proud of this financial success. We are equally aware of the part played by all our publications, both books and journals, in enhancing the Laboratory's worldwide reputation as a place of scientific excellence. With our current plans for growth and development, we intend both kinds of contributions to continue.

John R. Inglis

FINANCIAL STATEMENTS

LIABILITIES AND FUND BALANCES

	Operating Funds			Endowment & Similar Funds	Land, Building, & Equipment Funds	Total All Funds	
	Unrestricted						
	Undesignated	Designated	Restricted			1992	1991
Liabilities:							
Accounts payable and accrued expenses	$1,751,327	–	115,873	–	415,638	2,282,838	3,555,228
Notes payable	–	–	–	–	1,227,037	1,227,037	910,127
Bonds payable	–	–	–	–	20,000,000	20,000,000	20,000,000
Deferred revenue	1,156,836	–	2,402,284	–	–	3,559,120	3,845,730
Total liabilities	2,908,163	–	2,518,157	–	21,642,675	27,068,995	28,311,085
Fund balances:							
Unrestricted–undesignated	3,652,272	–	–	–	–	3,652,272	1,937,391
Unrestricted–designated	–	1,350,000	–	–	–	1,350,000	750,000
Endowment and similar funds	–	–	–	29,276,357	–	29,276,357	20,881,513
Land, buildings, and equipment:							
Expended	–	–	–	–	31,209,369	31,209,369	31,352,674
Unexpended–Donor restricted	–	–	–	–	291,723	291,723	1,079,986
Unexpended–Board authorized	–	–	–	–	2,079,639	2,079,639	1,457,746
Total fund balances	3,652,272	1,350,000	–	29,276,357	33,580,731	67,859,360	57,459,310
Total liabilities and fund balances	$6,560,435	1,350,000	2,518,157	29,276,357	55,223,406	94,928,355	85,770,395

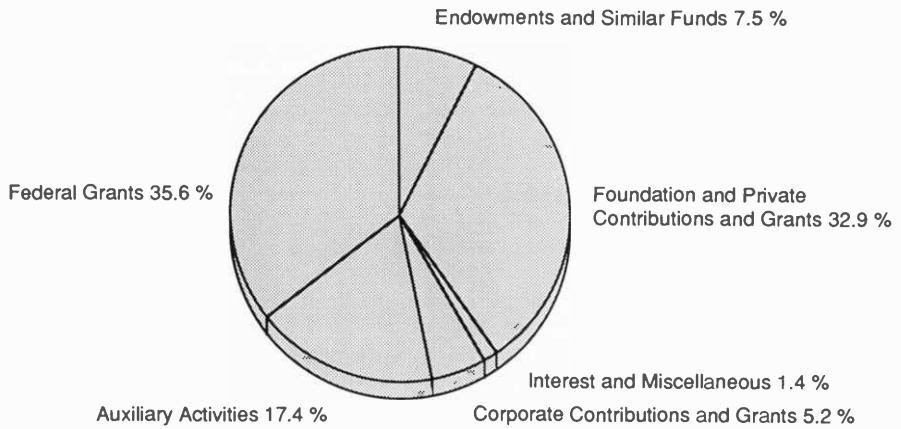
STATEMENT OF SUPPORT, REVENUE AND EXPENSES, AND CHANGES IN FUND BALANCES
Year ended December 31, 1992
with comparative amounts for 1991

	Operating Funds					Total All Funds	
	Unrestricted			Endowment &	Land, Building, &		
	Undesignated	Designated	Restricted	Similar Funds	Equipment Funds	1992	1991
Support and revenue:							
Public support	$1,869,338	–	7,633,686	7,302,098	551,885	17,357,007	13,116,508
Government grant awards	–	–	8,680,512	–	–	8,680,512	7,982,835
Indirect cost allowances	8,508,920	–	–	–	–	8,508,920	7,280,352
	10,378,258	–	16,314,198	7,302,098	551,885	34,546,439	28,379,695
Other revenue:							
Program fees	1,332,830	–	–	–	–	1,332,830	1,329,875
Rental income	298,104	–	–	–	–	298,104	278,840
Publications	3,709,326	–	–	–	–	3,709,326	3,078,938
Dining services	1,464,949	–	–	–	–	1,464,949	1,439,224
Rooms and apartments	1,320,424	–	–	–	–	1,320,424	1,239,585
Distribution from Robertson Funds	475,000	–	1,085,000	–	–	1,560,000	1,350,000
Investment income	139,666	–	–	1,966,523	9,847	2,116,036	1,973,581
Royalty & licensing	291,841	–	–	–	–	291,841	318,029
Miscellaneous	198,054	–	–	–	–	198,054	92,042
Total other revenue	9,230,194	–	1,085,000	1,966,523	9,847	12,291,564	11,100,114
Total support and revenue	19,608,452	–	17,399,198	9,268,621	561,732	46,838,003	39,479,809
Expenses:							
Program services:							
Research	–	–	12,985,969	–	–	12,985,969	13,026,253
Summer programs	939,106	–	3,814,480	–	–	4,753,586	2,994,584
Publications	3,547,638	–	–	–	–	3,547,638	3,488,100
Banbury Center conferences	201,740	–	249,261	–	–	451,001	447,044
DNA Education Center programs	12,054	–	485,667	–	–	497,721	379,804
Total program services	4,700,538	–	17,535,377	–	–	22,235,915	20,335,785
Supporting services:							
Direct research support	1,290,609	–	–	–	–	1,290,609	903,032
Library	468,569	–	–	–	–	468,569	433,496
Operation and maintenance of plant	4,575,804	–	–	–	–	4,575,804	4,221,594
General and administrative	3,101,996	–	–	114,029	–	3,216,025	3,081,287
Dining services	1,604,098	–	–	–	–	1,604,098	1,322,422
Interest	–	–	–	–	689,118	689,118	934,105
Total supporting services	11,041,076	–	–	114,029	689,118	11,844,223	10,895,936

Depreciation	–	–	–	–	2,357,815	2,357,815	1,898,421
Total expenses	15,741,614	–	17,535,377	114,029	3,046,933	36,437,953	33,130,142
Excess (deficiency) of support and revenue over expenses before designation	$3,866,838	–	(136,179)	9,154,592	(2,485,201)	10,400,050	6,349,667
Designation:							
Funds designated for research program	(600,000)	600,000	–	–	–	–	–
Excess (deficiency) of support and revenue over expenses and designation	3,266,838	600,000	(136,179)	9,154,592	(2,485,201)	10,400,050	6,349,667
Other changes in fund balances:							
Capital expenditures	(2,134,865)	–	(532,705)	–	2,667,570	–	–
Transfer to restricted funds	(150,000)	–	668,884	(518,884)	–	–	–
Transfer to endowment funds	(379,208)	–	–	871,252	(492,044)	–	–
Transfer to unrestricted funds	1,112,116	–	–	(1,112,116)	–	–	–
Net increase in fund balances	1,714,881	600,000	–	8,394,844	(309,675)	10,400,050	6,349,667
Fund balances at beginning of year	1,937,391	750,000	–	20,881,513	33,890,406	57,459,310	51,109,643
Fund balances at end of year	$3,652,272	1,350,000	–	29,276,357	33,580,731	67,859,360	57,459,310

Copies of our complete, audited financial statements, certified by the independent auditing firm of KPMG, Peat, Marwick & Co., are available upon request from the Comptroller, Cold Spring Harbor Laboratory.

SOURCES OF REVENUE
YEAR ENDED DECEMBER 31, 1992



COMPARATIVE OPERATING HISTORY
1988-1992
(Dollars in Thousands)

	1988	1989	1990	1991	1992
Income:					
Main Lab:					
Grants & contracts	10,799	13,062	13,535	15,172	16,800
Indirect cost reimbursement	5,707	6,412	6,558	7,170	8,388
Other	3,205	4,034	3,976	5,056	5,520
CSH Press	1,641	4,450	4,223	3,079	3,709
Banbury Center	976	1,012	1,120	1,090	1,104
DNA Learning Center	660	622	585	744	822
Total income	22,988	29,592	29,997	32,311	36,343
Expenses:					
Main Lab:					
Grants & contracts	10,799	13,062	13,535	15,172	16,800
Operation & maintenance of plant	3,010	3,412	3,759	3,904	4,241
General & administrative	2,102	2,377	2,414	2,468	2,634
Other	3,049	3,165	2,973	3,375	4,141
CSH Press	1,719	3,934	3,708	3,488	3,548
Banbury Center	910	1,038	1,125	1,063	1,070
DNA Learning Center	590	635	615	752	843
Total expenses	22,179	27,623	28,129	30,222	33,277
Excess before depreciation and designation of funds	809	1,969	1,868	2,089	3,066
Depreciation	(1,286)	(1,399)	(1,485)	(1,898)	(2,358)
Designation of funds (1)	–	(400)	(250)	(100)	(600)
Net operating excess (deficit)	$ (477)	170	133	91	108

The above amounts are presented on a combined basis for all funds for which Cold Spring Harbor Laboratory prepares operating budgets.

(1) Funds designated to underwrite future direct and indirect expenses of the neuroscience and other research programs.

FINANCIAL SUPPORT OF THE LABORATORY

FINANCIAL SUPPORT OF THE LABORATORY

Cold Spring Harbor Laboratory, Banbury Center, and the DNA Learning Center receive a substantial portion of their funding through grants from the Federal Government and through grants, capital gifts, and annual contributions from private foundations, corporations, and individuals. The following section summarizes funding that occurred during 1992.

GRANTS January 1, 1992–December 31, 1992

COLD SPRING HARBOR LABORATORY

Grantor	*Program/Principal Investigator*	*Duration of Grant*	*Total Award*
FEDERAL GRANTS			
NATIONAL INSTITUTES OF HEALTH			
Program Projects	Cancer Research Center, Dr. Mathews	1/92–12/96	$17,939,397*
	Cancer Center Support, Dr. Stillman	8/90–7/95	11,982,730
	Oncogene Program Project, Dr. Wigler	3/88–2/93	4,869,923
Research Support	Dr. Anderson	4/90–3/93	176,000
	Dr. Arndt	4/88–3/93	1,288,964
	Dr. Arndt	1/91–12/94	773,411
	Dr. Bar-Sagi	7/91–6/96	1,280,538
	Dr. Beach	12/84–1/93	1,481,482
	Dr. Beach	9/86–8/94	3,032,957
	Dr. Beach	7/88–6/93	1,488,740
	Dr. Beach	8/90–7/93	1,287,507
	Dr. Beach	9/91–8/95	847,368
	Dr. Davis	7/92–6/96	1,101,137*
	Dr. Davis	9/85–11/94	1,497,483
	Dr. Franza	10/91–8/94	487,949
	Dr. Franza	4/88–3/93	1,115,434
	Dr. Futcher	1/91–12/94	604,467
	Dr. Futcher	1/85–12/94	6,818,544
	Dr. Garrels	9/92–8/97	1,722,797*
	Dr. Gilman	12/89–11/94	1,130,019
	Dr. Greider	8/91–7/96	1,465,240
	Dr. Greider	9/85–3/94	2,109,264
	Dr. Helfman	7/92–6/96	1,441,344*
	Dr. Hernandez	9/91–8/96	1,141,822
	Dr. Hernandez	3/92–2/96	854,936*
	Dr. Herr	7/89–6/94	1,298,402
	Dr. Krainer	7/89–6/94	1,312,808
	Dr. Kuret	5/91–4/95	760,241
	Dr. Kuret	8/91–7/94	1,122,933
	Dr. Marr	8/92–7/94	245,421*
	Dr. Martienssen	2/92–1/97	1,396,361*
	Dr. Mathews	8/91–6/96	1,299,986
	Dr. Moran	8/91–5/95	1,122,557
	Dr. Moran	4/88–3/93	704,475
	Dr. Peterson	7/90–6/92	340,932
	Dr. Richards	7/88–6/93	1,598,876
	Dr. Roberts	9/88–8/92	681,311
	Dr. Roberts	4/91–3/94	652,140
	Dr. Roberts	4/90–3/95	1,441,475
	Dr. Spector	7/83–5/96	2,672,015
	Dr. Stillman	7/91–6/95	763,393
	Dr. Stillman	8/91–5/96	1,631,345
	Dr. Tonks	9/91–1/93	126,301
	Dr. Tully	7/92–6/99	13,298,000*
	Dr. Wigler	8/90–7/93	640,923
	Dr. Wigler		

* New Grants Awarded in 1992

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Equipment Support	Dr. Marshak	6/92–6/93	187,000 *
Fellowships	Dr. Ballester	2/89–1/92	63,966
	Dr. Brill	8/89–7/92	46,667
	Dr. Connolly	1/90–1/93	69,000
	Dr. Del Vecchio	12/91–11/93	22,700
	Dr. Grueneberg	10/90–9/93	69,000
	Dr. Jones	10/91–9/94	85,800
	Dr. Otto	9/90–8/92	67,720
	Dr. Regulski	8/91–6/92	49,658
	Dr. Prowse	9/92–8/94	55,500 *
	Dr. Skoulakis	9/92–8/94	48,300 *
	Dr. Szymanski	11/90–11/92	49,000
	Dr. Kwang-Ai Won	5/92–4/95	81,200 *
	Dr. Hwei-Gene Wang	8/92–7/93	55,500 *
	Dr. Zhang	9/92–8/97	416,558 *
Training Support	Institutional, Dr. Grodzicker	7/78–4/94	2,364,146
Course Support	Advanced Bacterial Genetics, Dr. Grodzicker	5/80–4/93	559,540
	Cancer Research Center Workshops, Dr. Grodzicker	1/83–3/92	1,010,057
	Neurobiology Short-term Training, Dr. Hockfield	5/82–4/96	1,300,588
	CSHL Analysis Large DNA Molecules, Dr. Grodzicker	1991–1996	475,538
	Essential Computational Genomics for Molecular Biologists, Dr. Marr	1991–1996	132,619
	Advanced In Situ Hybridization and Immunocytochemistry, Dr. Spector	1992–1997	228,841 *
Meeting Support	Genome Mapping and Sequencing Conference	1990–1993	78,040
	57th Symposium: The Cell Surface	1992	19,000 *
	Molecular Biology of Aging	1992	24,525 *
	Genetics and Molecular Biology of Breast Cancer	1992	11,000 *
	Mouse Molecular Genetics	1992	10,250 *
	RNA Processing	1992	2,000 *
NATIONAL SCIENCE FOUNDATION			
Research Support	Dr. Anderson	9/90–8/93	315,000
	Dr. Ma	5/91–4/93	399,000
	Dr. Ma	8/91–7/94	360,000
	Dr. Martienssen	7/89–6/92	305,000
	Dr. Peterson	11/91–4/93	207,212
	Dr. Roberts	2/90–2/93	210,000
	Dr. Sundaresan	12/92–11/95	140,000 *
Fellowship Support	Dr. Pena	6/92–5/95	4,600 *
Training Support	Undergraduate Research Program, Dr. Herr	6/91–5/94	150,000
Course Support	Plant Molecular Biology, Dr. Grodzicker	8/86–8/93	160,929
Meeting Support	RNA Processing Meeting	4/91–3/94	30,000
	57th Symposium: The Cell Surface	5/92–4/93	6,667 *
	Mouse Molecular Genetics	3/92–8/93	10,144 *

* New Grants Awarded in 1992

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
DEPARTMENT OF ENERGY			
Research Support	Dr. Marr	7/91–2/94	814,022
	Dr. Martienssen	8/91–8/93	144,000
	Dr. Peterson	5/92–5/95	291,000 *
Meeting Support	57th Symposium: The Cell Surface	1992	15,000 *
	Genetics and Molecular Biology of Breast Cancer	1992	10,150 *
OFFICE OF NAVAL RESEARCH			
Course Support	Training in Computational Neuroscience at CSHL, Dr. Hockfield	6/92–5/93	32,815*
U.S. DEPARTMENT OF AGRICULTURE			
Research Support	Dr. Sundaresan	8/91–8/94	221,000
	Dr. Ma	9/92–9/94	138,000 *
	Dr. Martienssen	9/91–8/93	128,000
Course Support	Support of a Workshop on Molecular Markers for Plant Breeding, Dr. Sundaresan	10/92–9/93	35,015 *
NONFEDERAL GRANTS			
Research Support			
Allied Signal	Dr. Greider	11/92–10/93	100,000 *
American Cancer Society	Dr. Gilman	7/91–5/94	300,000
	Dr. Gilman, Institutional	7/90–6/92	100,000
	Dr. Spector	7/91–6/92	50,000
	Dr. Sutton	1/92–12/93	190,000 *
	Dr. Wigler, Professorship	1986–2012	1,333,333
	Dr. Wigler	4/92–3/93	10,000 *
American Heart Association	Dr. Helfman	7/91–6/96	175,000
Amersham International plc	Dr. Krainer	11/86–10/92	827,925
American Foundation for AIDS Research	Dr. Laspia	12/91–11/93	120,000
	Dr. Mathews	10/91–9/93	120,000
Arab Republic of Egypt	Dr. Marshak	11/91–10/94	48,907 *
Argonne National Laboratories	Dr. Pflugrath	2/91–8/93	192,232
	Dr. Pflugrath	1/92–12/92	100,000 *
Sara Chait Foundation	Dr. Marshak	12/91–11/96	125,000
Council for Tobacco Research	Dr. Greider	7/92–6/93	52,000 *
	Dr. Helfman	7/91–6/93	160,000
	Dr. Spector	7/92–6/93	80,000 *
Garfield Foundation	AIDS Research	12/91–5/93	200,000
Greenwall Foundation	Dr. Marshak	7/92–6/93	70,439 *
Geron Corporation	Dr. Greider	8/92–8/95	225,000 *
Irving A. Hansen Memorial Foundation	Dr. Tonks	7/92–6/94	20,000 *
Hitachi Foundation	Dr. Nawa	3/92–2/94	10,000 *
Hechler Private Investments	Dr. Enikolopov	12/91–5/92	27,812
Howard Hughes Medical Institute	Neurobiology Support	1987–1992	1,000,000
Human Frontier Science Program	Dr. Futcher	6/92–5/96	183,986 *
	Dr. Davis	5/92–4/95	147,935 *
ICOS Corporation	Dr. Futcher	7/92–6/94	126,995 *
	Dr. Tonks	4/92–3/94	227,842 *

* New Grants Awarded in 1992

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
ICI Seeds	Dr. Martienssen/Dr. Richards	1/92–12/94	41,676*
Johnson & Johnson	Dr. Skowronski	5/92–4/95	270,000*
Esther and Joseph A. Klingenstein Fund, Inc.	Dr. Nawa	7/92–6/95	100,000*
Lucille P. Markey Charitable Trust	Neurobiology Support	7/90–6/96	
	Neuroscience Building Fund		2,000,000
			2,000,000
Mathers Charitable Foundation	Dr. Davis	8/91–7/94	1,028,000
Mellam Family Foundation	Dr. Franza	12/88–11/95	350,000
Richard Meltzer Fund for Cancer Research (World Business Council)	Dr. Stillman	3/90–2/92	50,000
John Merck Fund	Dr. Tully	9/91–5/94	180,000
Massachusetts Institute of Technology (Subcontract)	Dr. Marr	11/91–7/92	49,006
Muscular Dystrophy Association	Dr. Helfman	1/91–12/93	126,100
National Down's Syndrome Society	Dr. Marshak	7/90–6/92	50,000
NYU Consortium with NIH	Dr. Marshak	5/92–4/97	886,546*
Oncogene Science, Inc.	Monoclonal Agreement	6/92–5/95	300,000*
Oxnard Foundation	Dr. Gilman	2/91–1/94	150,000
Pew Memorial Trust	Dr. Greider	7/90–6/94	200,000
	Dr. Krainer	7/92–6/96	200,000*
	Dr. Tonks	7/91–6/95	200,000
	Dr. Peterson	8/91–7/94	150,000
Pioneer Hi-Bred International Inc.			
Rita Allen Foundation	Dr. Hernandez	10/89–9/94	150,000
Samuel Freeman Charitable Trust	Freeman Laboratory of Cancer Cell Biology	7/89–6/94	1,000,000
Equipment Support			
Florence Gould Foundation	Neuroscience Equipment	7/91–6/94	300,000
Fellowships			
American Cancer Society	Dr. Caligiuri	1/91–12/93	69,000
	Dr. Flanagan	7/92–6/95	78,000*
	Dr. Hinkley	9/92–8/95	72,000*
	Dr. Mi Sha Jung	8/92–7/94	54,000*
	Dr. Lundgren	1/92–12/92	28,000*
	Dr. Nefsky	7/90–6/93	69,000
American Foundation for AIDS Research	Dr. Scheppler	7/91–6/94	112,000
American Heart Association	Dr. Kazzaz	1/92–12/93	60,000*
Cancer Research Institute	Dr. Flint	1/92–12/95	87,000*
Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation	Dr. Hannon	1/92–12/94	84,000*
	Dr. Hardy	9/92–8/95	84,000*
	Dr. Horton	9/92–8/95	84,000*
	Dr. V. Jung	5/92–4/95	84,000*
	Dr. Ruppert	8/91–7/94	84,000
	Dr. Sun	5/92–4/95	84,000*
	Dr. Walworth	9/90–8/93	84,000
	Dr. Wilson	1/91–12/93	69,000
Ford Foundation	Dr. Pena	9/91–5/92	30,000

* New Grants Awarded in 1992

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Government of Canada	Dr. Demetrick	11/91–10/93	6,137
Joyce Green Family Foundation	Dr. Sun	12/91–2/92	5,000
Helen Hay Whitney Foundation	Dr. Bell	7/91–6/94	75,000
Human Frontier Science Program	Dr. Alexandre	1/92–12/93	84,560 *
	Dr. Clarke	7/91–6/93	76,000
	Dr. Mayeda	7/91–6/93	77,000
	Dr. Tansey	9/92–9/94	68,216 *
Irvington Institute for Medical Research	Dr. Giordano	7/90–6/92	4,000
The Leukemia Society of America	Dr. Das	7/89–6/92	76,140
	Dr. Bischoff	7/91–6/94	76,140
CSHL Association	Fellowships	4/92–3/93	125,000 *
Life Science Research Foundation	Dr. D. Roberts	9/89–8/92	90,000
	Dr. Collins	6/92–5/95	105,000 *
	Dr. Kaufman	7/92–6/95	105,000 *
Muscular Dystrophy Association	Dr. Tsukahara	7/91–6/92	25,000
Andrew Seligson Memorial Fellowships	Cancer Research Fellowships	9/90–5/93	145,000
Training Support			
Anonymous	Summer Undergraduate Program	1992	18,750 *
Baring Brothers & Co., Ltd.	Summer Undergraduate Program	1990–1992	75,233
Bio-Rad Laboratories	Summer Undergraduate Program	1992	1,000 *
Bliss Fund	Summer Undergraduate Program	1992	4,000 *
Burroughs Wellcome Foundation	Summer Undergraduate Program	1991–1993	49,200
Theodore Danforth	Summer Undergraduate Program	1992	2,000 *
Grass Foundation	Neurobiology Scholarship Support	1980–1992	201,030
ICI	Summer Undergraduate Program	1992	7,441 *
Phillips Petroleum Co.	Summer Undergraduate Program	1992	1,000 *
Robert H.P. Olney Memorial Fund	Undergraduate Research Program	1992	5,000 *
Hansen White Foundation	Summer Undergraduate Program	1991	6,400 *
Course Support			
Howard Hughes Medical Institute	Postgraduate Courses	1991–1995	1,000,000
Esther and Joseph A. Klingenstein Fund, Inc.	Advanced Neurobiology Courses	3/91–2/94	180,000
Meeting Support			
Boehringer Mannheim Co.	Regulation of Liver Gene Expression and Disease	1992	250 *
Clontech Laboratories	Regulation of Liver Gene Expression and Disease	1992	300 *
Council for Tobacco Research	Cytoskeleton Cell Function	1992	1,000 *
ICN Biomedicals	Translational Control Meeting	1992–1993	20,000 *
Esther and Joseph A. Klingenstein Fund, Inc.	Epilepsy Meeting	1992–1993	96,537 *
March of Dimes	Cytoskeleton Cell Function	1992–1993	4,000 *
Miles, Inc.	Regulation of Liver Gene Expression and Disease	1992	1,000 *
Wellcome Trust	Gene Therapy Meeting	1992	43,175 *

* New Grants Awarded in 1992

DNA LEARNING CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
NATIONAL SCIENCE FOUNDATION			
	High School Faculty Enhancement	1990–1993	489,036
	Middle School Faculty Enhancement	1990–1993	252,614
	College Faculty Enhancement	1991–1993	264,467
U.S. DEPARTMENT OF EDUCATION			
	College Faculty Enhancement	1991–1993	170,033
NONFEDERAL GRANTS			
Cablevision Systems Corporation	Capital Support	1992	250,000*
Stone Foundation	Program Support	1991–1994	250,000
Howard Hughes Medical Institute	High School Faculty Enhancement	1990–1993	46,500
Henderson State University	High School Faculty Enhancement	1992	2,472*
University of Kentucky, Lexington	High School Faculty Enhancement	1992	2,240*
University of Nevada, Reno	High School Faculty Enhancement	1992	2,835*
Abell Foundation	Minority and Special Programs	1992	2,100*
Barker Welfare Foundation	Minority and Special Programs	1992	5,000*
Brinkmann Instruments	Minority and Special Programs	1992	1,000
Corporate Advisory Board	Minority and Special Programs	1992	14,500*
Harweb Foundation	Minority and Special Programs	1992	2,000*
Laurie Landeau	Minority and Special Programs	1992	4,000
Life Technologies	Minority and Special Programs	1992	500
SUNY at Stony Brook	Minority and Special Programs	1992	10,000*
Edwin S. Webster Foundation	Minority and Special Programs	1992	15,000
Italian Ministry of Research	Exhibit Support	1992	45,450
The Weezie Foundation	Exhibit Support	1991–1992	100,000
New York State Legislature	Middle School Program	1992	50,000*
William Randolph Hearst Foundation	Middle School Program	1991–1994	100,000
Commack Union Free School District	Whole Learning Program	1992	5,000*
Half Hollow Hills Central School District	Whole Learning Program	1992	5,000*
Locust Valley Central School District	Whole Learning Program	1992	5,000*
Plainedge Union Free School District	Whole Learning Pogram	1992	5,000*
Commack Union Free School District	Curriculum Study	1992	500
East Williston Union Free School District	Curriculum Study	1992	500
Elwood Union Free School District	Curriculum Study	1992	2,000*
Garden City Union Free School District	Curriculum Study	1992	500
Great Neck Public Schools	Curriculum Study	1992	500
Half Hollow Hills Central School District	Curriculum Study	1992	500
Harborfields Central School District	Curriculum Study	1992	500
Herricks Union Free School District	Curriculum Study	1992	500
Island Trees Union Free School District	Curriculum Study	1992	500
Jericho Union Free School District	Curriculum Study	1992	500
Kings Park Central School District	Curriculum Study	1992	500
Lawrence Union Free School District	Curriculum Study	1992	500
Lindenhurst Union Free School District	Curriculum Study	1992	500
Locust Valley Central School District	Curriculum Study	1992	500
Manhasset Union Free School District	Curriculum Study	1992	500
Massapequa Union Free School District	Curriculum Study	1992	1,500

* New Grants Awarded in 1992

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Northport-East Northport Union Free School District	Curriculum Study	1992	500
North Shore Central School District	Curriculum Study	1992	500
Oyster Bay-East Norwich Central School District	Curriculum Study	1992	500
Plainview-Old Bethpage Central School District	Curriculum Study	1992	500
Plainedge Union Free School District	Curriculum Study	1992	500
Portledge School	Curriculum Study	1992	500
Port Washington Union Free School District	Curriculum Study	1992	500
Roslyn Public Schools	Curriculum Study	1992	500
Sachem Central School District at Holbrook	Curriculum Study	1992	500
South Huntington Union Free School District	Curriculum Study	1992	500
Syosset Central School District	Curriculum Study	1992	500

BANBURY CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
DEPARTMENT OF ENERGY			
	Human Genetics and Genome Analysis: Practical Workshop for Public Policy Makers and Opinion Leaders	3/91 -3/93	128,059
	DNA Repeats and Human Gene Mutations	9/92 -9/93	15,000 *
NONFEDERAL SUPPORT			
Meeting Support		1992	1,000 *
Abbott Laboratories	Constructing Organisms	1993	5,000 *
Abbott Laboratories	Lyme Disease Symposium	1993	10,000 *
Allen & Hanburys (Glaxo, Inc.)	Lyme Disease Symposium	1992	27,200 *
The Charles A. Dana Foundation	Decade of the Brain	1991	2,000 *
Dr. Amy and James Elster Research Fund	Marfan Meeting	1992	2,000 *
Hoffmann-La Roche Inc.	Control of HIV Expression	1993	10,000 *
Pfizer Inc.	Lyme Disease Symposium	1992	4,000 *
Merck Research Laboratories	Control of HIV Expression	1992	5,000 *
RepliGen Corporation	Constructing Organisms	1992	5,000 *
RepliGen Corporation	Control of HIV Expression	1991	2,500 *
Sandoz Corporation	Marfan Meeting	1990 -1992	150,000
Alfred P. Sloan Foundation	Journalists and Congressional Workshops	1992	19,579 *
The Wellcome Trust	Contructing Organisms	1991 -1993	150,000
The William Stamps Farish Fund	Meetings on Complex Genetic Diseases		

* New Grants Awarded in 1992

CORPORATE SPONSOR PROGRAM

Restricted

The Corporate Sponsor Program provides support for the meetings held in both Grace Auditorium and Banbury Center. It is generally agreed that the Laboratory's meetings program is second to none in the world both in the topics selected for discussion and in the quality of the meetings themselves. That we are able to maintain such an excellent series is due in large measure to the stability provided by the continuing generosity of the companies who are members of the Program. In 1992, the number of members increased from 32 to no fewer than 38; these companies are listed below.

In acknowledgment of our members' contributions, we waive all on-site fees for eight representatives of each company at our meetings. Three of these scientists may attend meetings at the Banbury Center, where attendance is otherwise only by invitation of the organizers. Furthermore, Corporate Sponsors receive gratis copies of all publications of the Cold Spring Harbor Laboratory Press. These include the journal *Genes & Development*, which has ranked first among cell and developmental biology journals for two consecutive years, and *PCR*, which received an enthusiastic review in *Nature*. These benefits add up to a package that accounts for a substantial part of the membership fee.

In addition, our Sponsors are acknowledged in all relevant publications, abstract booklets, and other materials published by Cold Spring Harbor Laboratory Press. We list the Corporate Sponsors' names on the meeting poster and in 1992, we included a specially designed poster in the package given to the more than 6000 scientists who attended our meetings. We and all those scientists thank our Corporate Sponsors for their commitment to scientific excellence.

Total Annual Contributions **$732,875**

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CAPITAL GIFTS
Second Century Campaign

January 1, 1986–December 31, 1992

As part of the Laboratory's Centennial celebration in 1990, the public phase of a major fund-raising campaign—the Second Century Campaign—was organized, with a goal of raising a total of $44 million in support of construction, endowment, and program projects of the Laboratory. At the end of 1992, the Campaign had raised $51,092,883, including $1,538,100 of net interest. The very important net effect of this Campaign has been to expand the Laboratory's capacity to pursue science and to strenghten its financial structure.

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Net Interest Earned 12/31/92	**1,538,100**
Total	**$51,092,883**

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ANNUAL CONTRIBUTIONS

Summary of Annual Contributions

Unrestricted Annual Contributions

CSHLA Major Gifts	$347,279	
CSHLA Memberships	63,691	
Memorials	25,570	
		$436,540

Restricted Annual Contributions	**$94,723**
Corporate Advisory Board—DNA Learning Center	**$29,100**
Undergraduate Research Program	**$128,404**
Total Annual Contributions	**$688,767**

Annual unrestricted contributions are donations from members of the Cold Spring Harbor Laboratory Association. In 1992, these totaled $436,540 from 896 members.

Major Gifts ($1,000–$10,000) accounted for $347,279 of this total from 173 members. Major givers (Associates, Patrons, Benefactors) participate in a program of research briefings, guest lectures, and "hands-on" laboratory workshops and are invited to the annual Associates cocktail party. Membership in the Cold Spring Harbor Laboratory Association, which sponsors a program of lectures and other activities at the Laboratory, requires an annual contribution of $50 or more.

For the past 5 years, the Association has provided funds for postdoctoral fellows who work under the guidance of senior laboratory scientists. The 1992 fellowship recipients were Judy Wang, Anthony Rossamando, Gert Bolwig, and Keiko Mizuno. The Annual Fund provides also New Investigator Start-up Funding to new staff members for purchase of equipment or supplies. This year's recipients were Yi Zhong and Alcino Silva.

In addition to the Annual Fund of the Cold Spring Harbor Laboratory Association, the Laboratory received $94,723 of restricted annual contributions and $128,404 for support of the Undergraduate Research Program from a variety of corporate and individual sources. The Annual Fund of the DNA Learning Center, which is sponsored by the Corporate Advisory Board, raised $29,100 in 1992; Douglas B. Fox is Chairman of the Corporate Advisory Board. Total annual contributions for 1992 were $688,767.

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PRESIDENT'S REPORT

1992 was an active year for the Cold Spring Harbor Laboratory Association, measured in terms of number of events sponsored for the community and the annual giving support provided for the Laboratory.

Annual Giving swelled to $531,263, a new record. There were 173 *Associates* (donors of $1000 or more), including 33 *Patrons* (donors of $3000 to $9999), and 10 *Benefactors* (donors of $10,000 or more). There were 72 *Friends* (donors of $300 to $999), 525 *Family Members* (contributors of $50 to $299), and another 60 contributors of lesser amounts.

The Association Directors, Laboratory Administration, and, most importantly, the scientists themselves thank all of the donors for their support. Contributions of all sizes played important roles in achieving the success of our annual giving effort.

Another source of Annual Fund support has been Memorial Contributions (see Memorial Contributors at end of section). In 1992, gifts were made in memory of

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Dorothy Zehner

Calendar of Events

1992 began with our Annual Meeting on January 12th. Drs. Ron Davis and Tim Tully, who use fruit flies to study learning and memory, gave a talk on neuroscience and their particular research efforts. Gert Bolwig, a young Ph.D. in Ron Davis' laboratory, was the recipient of a 1992 Association Fellowship award.

On January 25th, Peter Orth returned to give a piano recital, again to a full house. His exciting playing dazzled the crowd of members and friends. March 27th was the evening we had a dinner at the Piping Rock Club, involving several members of the scientific staff, which resulted in a wonderful informal discussion about science.

In April, we held a DNA workshop for Major Givers at the DNA Learning Center. These events give our members a real idea of what basic research is like, particularly since they can see first hand that some experiments fail while others succeed. This year, workshops will be made available to *Friends*, as well as *Associates*, *Patrons*, and *Benefactors.*

Barbara Conolly, a leading ornithologist, led a nature walk along the shoreline and through our campus grounds for Major Givers on May 2nd. Her expert eyes helped us to identify many birds and trees that we were not aware existed on the Laboratory grounds. The following afternoon, the Laboratory sponsored an enthusiastically received violin recital by Scott Yoo for visiting scientists and Association members.

On May 31st, Dr. Michael Brown became the third consecutive Nobel prize winner to address the Dorcas Cummings Memorial Lectures. He had excellent slides and spoke so clearly that most of us came away with a much better understanding about the process of exchange of information from one cell to another. Following the lecture, many of the scientists, who were attending the Laboratory's Symposium on the Cell Surface, went to 19 dinner parties hosted by community members of the Association. They were joined by members of our scientific staff, as well as friends of the hosts. The hosts and hostesses were

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September got off to an auspicious start. Drs. Mary Claire King and Mark Lippman conducted a panel discussion on breast cancer the day before they led a Banbury conference on this topic for scientists from around the country. It seemed appropriate to bring our membership up to date as well on recent progress. Since their panel discussion was scheduled for a Tuesday evening and a good attendance was sought, an order was sent out to your President in Alaska to provide a dinner featuring native Alaskan treats. He was able to catch enough halibut to feed all the attendees in the newly expanded Blackford Hall. One might say that the membership "rose to the bait" as Grace Auditorium was filled! The progress on breast cancer is exciting, with Laboratory scientists playing important roles. Every effort will be taken to keep the community up to date on the progress of this research effort.

The Sutherlands address the Association.

The annual cocktail party was held on October 8th, following the dedication of the Racker Reading Room in Blackford Hall. In attendance were Laboratory Trustees, Association Directors, and Alumni from the 1940s and 1950s, as well as many of our scientific staff.

October 18th was another exciting day in the Association's calendar of events. Dr. Michael Novacek, Director of Science for the American Museum of Natural History, spoke to the membership on his recent expeditions to the Gobi Dessert in search of dinosaur fossils.

On November 7th, a panel discussion on Alzheimer's disease organized by Dr. Daniel Marshak was jointly sponsored by the Harvard Club of Long Island and the Association. Finally, on November 5th, we were very fortunate to have Dr. Thomas Sutherland and his wife address our members. Dr. Sutherland came at Dr. Watson's invitation. He is the former director of the Biology Department at the American University of Beirut and was a hostage for more than 5 years. The strength of these two extraordinary people was apparent from the start and the evening was certainly one of the highlights of the year.

In summation, the programs in 1992 for members and our scientific staff were highly diversified and surely touched upon the varied interests of all.

As I step down as Association President after 7 years, I do so with confidence that the Association will continue to support the young scientists and offer an informative program for its membership. The Board of Directors has done an extraordinary job of building the level of support to where it stands today. Every Director has been an active participant in our effort, and each has contributed to the 1992 Annual Fund. The aggregate contribution from Directors has now reached $60,000!

The Annual Fund has become a very important part of the Laboratory's funding of science programs and, although it reached well over $500,000 in 1992 for the first time, we must seek higher levels to meet the Laboratory's needs. Our most effective Development Department staff and Association Directors depend on the members themselves to build Annual Fund support to ensure that science at Cold Spring Harbor maintains its excellence through the continued infusion of highly qualified young scientists.

George W. Cutting, President

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