FRONT COVER

Fluorescence photograph of a whole mount of *Drosophila melanogaster* embryo (0.42 mm x 0.15 mm), approximately 14 hours after fertilization, stained with monoclonal antibody MAb3B4. This ventral view reveals specific surface structures. The three pairs of bright spots in the thoracic segments (top) are Keilin's organs. Also, the abdominal setal belts are stained. Internally, the gut and yolk are faintly autofluorescent. Preparation was made by John Pollock, using one of the monoclonal antibodies previously isolated in Seymour Benzer's laboratory using *Drosophila* head, brain or retina as immunogens. (See abstracts by the Benzer group starting on page 201.)

BACK COVER

Stripes in visual cortex. Visual area V2 in monkeys contains an array of stripe-like subregions that can be visualized by histochemical staining for the enzyme cytochrome oxidase. The lower panel shows the pattern of stripes on a composite drawing of flattened sections of V2, cut parallel to the cortical surface. Alternating thick and thin stripes (dark arrows) are separated by pale interstripes (open arrows). The upper panel shows results from injections of retrogradely-transported fluorescent tracers into target areas V2 and MT. Cells projecting to MT (blue) coincide with thick stripes, whereas cells projecting to V4 (red) occupy both thin stripes and interstripes. Illustration by E. DeYoe and K. Tazumi. (See abstracts by the Van Essen group starting on page 191.)
A REPORT FOR THE YEAR 1984-85
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
AT THE
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA
Constance R. Katz

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A special thanks to Bernita, a very special person who gave us so much. We miss you.

RESEARCH REPORTS

Much of the research work summarized here has not yet been reported in print, in many instances because it is not yet complete. For that reason this report is not intended as a publication and should not be cited as such. Individual projects should be referred to only if specific permission to do so is obtained from the investigator responsible for the material. References are made here to published papers bearing on the projects reported. Publications by members of the Division, covering the period April 1984-June 1985, are listed separately at the end of the research reports of each group.
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INTRODUCTION
The Faculty

Two additions to the faculty of the Division of Biology were made this year:

James Bower arrived in January to take up his appointment as Assistant Professor of Biology. Jim is a Montanan who received his undergraduate education at Antioch College and Montana State University and then moved to the University of Wisconsin where he obtained his Ph.D. in 1981. At Wisconsin, Jim began his work on the neurophysiology of the cerebellum, which led him to iconoclastic ideas about how this part of the brain works. He then spent two years as a postdoc with Rodolfo Llinas in the Department of Physiology and Biophysics at NYU before returning to Wisconsin. Jim has moved into laboratories on the third floor of the Beckman Laboratories for Behavioral Biology and has already begun important collaborations with other neurobiologists in Beckman, combining his electrophysiological techniques with more molecular methods.

Judy Campbell became a joint Associate Professor of Chemistry and Biology this year. Judy, of course, is not a newcomer, having been a member of the faculty of the Division of Chemistry and Chemical Engineering since 1977 and a major participant in Biology Division affairs such as chairmanship of the General Biology Seminar Committee and service on the Graduate Admissions Committee in Biology for several years. Her research on the enzymatic mechanisms of DNA replication in yeast is also equally at home in our Division or in the group of chemical biologists within the Chemistry Division. We are happy to formalize her interactions with our Division by this joint appointment.

Bela Julesz, from Bell Laboratories, spent the winter term in residence at Caltech as a Visiting Professor. This is expected to be a regular event, as part of a long-term collaboration with Profs. John Allman and David Van Essen and other members of our neurobiology faculty group. Julesz is of course widely known for his contributions to the understanding of human visual perception.

Joan and Tom Steitz, from Yale University, spent 6 months at Caltech as Fairchild Scholars in the Division of Biology, working in association with Prof. John Abelson.

Doug Fambrough, who received his Ph.D. in Biology from Caltech in 1968 and now holds a position at the Carnegie Institution of Washington, returned this year for a three-month visit as the first Wiersma Visiting Professor in Neurobiology. This visiting professorship was established by the bequest of Prof. C. A. G. Wiersma, following his death in 1979.
Honors and Awards

The week of April 21, 1985 began with the announcement of the election of four members of the Division faculty to the National Academy of Sciences—Professors John Abelson, Eric Davidson, Mark Konishi, and Melvin Simon. This extraordinary event was marked by a spur-of-the-moment celebration in the Alles Patio on April 23, hosted by the Biology Division.

Later in the same week, Division Chairman Leroy Hood was named California Scientist of the Year at a banquet for the California Museum of Science and Industry. This prestigious award, which also carried with it a cash prize of $5000, was the result of Lee's selection by a nine-member jury chaired by Prof. Paul Saltman (Ph.D., 1953) of the University of California at San Diego.

Professors John Abelson and Howard Berg were elected to the American Academy of Arts and Sciences.

Professor Giuseppe Attardi was named the Grace C. Steele Professor of Molecular Biology.

Professor Seymour Benzer was elected to Honorary Life Membership of the New York Academy of Sciences.

Assistant Professor Scott Emr was selected for a Presidential Young Investigator Award from NSF.

Professor Leroy Hood also received the Bertner Memorial Award at the University of Texas at Houston.

Visiting Professor Bela Julesz received the Heineken Prize, awarded in Holland for his research on visual psychophysics.

Associate Professor Mary Kennedy received a McKnight Neuroscience Development Award.

Professor Mark Konishi received a Javits Neuroscience Investigator Award from NIH, providing 7 years of research support, in recognition of his distinguished record for substantial contributions to neuroscience.

Professor Paul Patterson received the W. Alden Spencer Award in Developmental Neurobiology from Columbia University and a McKnight Neuroscience Research Project Award.

Donna Livant, who received her Ph.D. in Biology in June 1985, was a winner of this year's Milton and Francis Clauser Doctoral Prize at Caltech for a Ph.D. thesis exhibiting originality and the potential for opening up new avenues of human thought. Biology Ph.D.s have won this prize in 4 out of the past 5 years! Congratulations, Donna.
Special Lectures

The 1985 Jean Weigle Memorial Lecture was presented on May 7 by Professor Gunther Stent from the University of California at Berkeley. He spoke on "Meaning in Art and Science."

Visiting Professor Doug Fambrough, the first Wiersma Visiting Professor, presented a memorial lecture on May 16 on the topic "Regulation of the Sodium Pump in Nerve and Muscle."

In Memoriam

Bernita (Larsh) Lewis, a long-time member of the secretarial staff of the Division, and most recently Division secretary for Lee Hood, passed away on February 10, 1985, following a valiant struggle with cancer. Bernita's friends gathered together on the morning of March 28 to memorialize her by planting a Chinese magnolia tree alongside the walk east of the Braun Laboratory. Bernita's cheerful willingness to help in the face of the pressures of her job and her terminal illness will be remembered as a model for all of us to emulate.

We are also sad to note the death of Daniel McMahon in Pasadena during June 1985. Dan was Assistant Professor of Biology from 1968 to 1975 and will be remembered by several generations of students as an enthusiastic and popular teacher.
STAFF OF INSTRUCTION AND RESEARCH

Division of Biology

Leroy E. Hood, Chairman
Charles J. Brokaw, Associate Chairman
David C. Van Essen, Executive Officer
James H. Strauss, Executive Officer

Professors Emeriti

James F. Bonner, Ph.D.
Biology
Sterling Emerson, Ph.D.
Genetics
Norman H. Horowitz, Ph.D.
Biology
George E. MacGinitie, M.A.
Biology
Herschel K. Mitchell, Ph.D.
Biology
Ray D. Owen, Ph.D., Sc.D.
Biology
Roger W. Sperry, Ph.D., Sc.D., Nobel Laureate
Board of Trustees Professor Emeritus of Psychobiology
Anthonie Van Harreveld, Ph.D., M.D.
Physiology

Professors

John N. Abelson, Ph.D.
Biology
John M. Allman, Ph.D.
Biology
Giuseppe Attardi, M.D.
Grace C. Steele Professor of Molecular Biology
Seymour Benzer, Ph.D., D.Sc.
James G. Boswell Professor of Neuroscience
Howard C. Berg, Ph.D.
Biology
Charles J. Brokaw, Ph.D.
Biology
Eric H. Davidson, Ph.D.
Norman Chandler Professor of Cell Biology
William J. Dreyer, Ph.D.
Biology
Derek H. Fender, Ph.D.
Biology and Applied Science
Leroy E. Hood, M.D., Ph.D.
Ethel Wilson and Robert Bowles Professor of Biology
John J. Hopfield, Ph.D.
Roscoe G. Dickinson Professor of Chemistry and Biology
Masakazu Konishi, Ph.D.
Bing Professor of Behavioral Biology
Elias Lazarides, Ph.D.
Biology
Henry A. Lester, Ph.D.
Biology
Edward B. Lewis, Ph.D.
Thomas Hunt Morgan Professor of Biology
Paul H. Patterson, Ph.D.
Biology
Jean-Paul Revel, Ph.D.
Albert Billings Ruddock Professor of Biology
Melvin I. Simon, Ph.D.
Biology
James H. Strauss Jr., Ph.D.
Biology
David C. Van Essen, Ph.D.
Biology
Elliot M. Meyerowitz, Ph.D.
Biology

Associate Professors

Judith L. Campbell, Ph.D.
Chemistry and Biology
Mary B. Kennedy, Ph.D.
Biology

Assistant Professors

James M. Bower, Ph.D.
Biology
Scott D. Emr, Ph.D.
Biology
Ellen Rothenberg, Ph.D.
Biology
Mark A. Tanouye, Ph.D.
Biology
Barbara J. Wold, Ph.D.
Biology

Visiting Professors

Douglas M. Fambrough, Ph.D.
Biology
Bela Julesz, Ph.D.
Biology
Distinguished Sherman Fairchild Scholars

Joan A. Steitz, Ph.D.

Biology

Thomas A. Steitz, Ph.D.

Biology

Distinguished Carnegie Senior Research Associate

Roy J. Britten, Ph.D.

Biology

Senior Research Associates

Charles R. Hamilton, Ph.D.

Biology

Barbara Hough-Evans, Ph.D.

Biology

Stephen B. H. Kent, Ph.D.

Biology

Marianne E. Olds, Ph.D.

Biology

Ellen G. Strauss, Ph.D.

Biology

S. Barbara Yancey, Ph.D.

Biology

Senior Research Fellows

Richard K. Barth, Ph.D.

Biology

Yassemi G. Capetanaki, Ph.D.

Biology

Anne Chomyn, Ph.D.

Biology

Constantin N. Flytzanis, Dr.rer.nat

Biology

Shahid M. M. Khan, Ph.D.

Biology

Nancy Lan, Ph.D.

Biology

W. James Nelson, Ph.D.

Biology

Jeanne M. Nerbonne, Ph.D.

Biology

Andrew J. Newman, Ph.D.

Biology

John W. Roberts, Ph.D.

Biology

Samuel J. Ross III, Ph.D.

Biology

Lloyd M. Smith, Ph.D.

Biology

Iwona Stroynowski, Ph.D.

Biology

Martha C. Zuniga, Ph.D.

Biology

Myron A. Bantrell Research Fellows

Dennis G. Ballinger, Ph.D.

Nathan Dascal, Ph.D.

Research Fellows

Ruedi Aebersold, Ph.D.

Bernhard Arden, Ph.D.

Utpal Banerjee, Ph.D.

Vytais A. Bankaitis, Ph.D.

William V. Bleisch, Ph.D.

Steven M. Block, Ph.D.

V. Craig Bond, Ph.D.

Michael P. Bruist, Ph.D.

Frank J. Calzone, Ph.D.

Barry L. Caplan, Ph.D.

Catherine E. Carr, Ph.D.

Susan E. Celniker, Ph.D.

Lee D. Chabala, Ph.D.

Sandra P. Chang, Ph.D.

Soo-Chen Cheng, Ph.D.

Hilde Cheroutre, Ph.D.

Ariene Y.-C. Chu, Ph.D.

Michael W. Clark, Ph.D.

Ian Clark-Lewis, Ph.D.

Pat Concannon, Ph.D.

Nancy A. Costiow, Ph.D.

John V. Cox, Ph.D.

Alice M. Cronin-Golomb, Ph.D.

Michael E. Cusick, Ph.D.

Gloria Dalbadie-McFarland, Ph.D.

Edgar DeYoe, Ph.D.

Claus-Jens W. Doersen, Ph.D.

Susan J. Eberlein, Ph.D.

Daniel J. Felleman, Ph.D.

Henry K. W. Fong, Ph.D.

Roberta R. Franks, Ph.D.

Karl J. Fryxell, Ph.D.

Keiko Fukada, Ph.D.

Janet M. Garrett, Ph.D.

Joan M. Goverman, Ph.D.

Bruce L. Granger, Ph.D.

Phillip R. Green, Ph.D.

Alison M. Gurney, Ph.D.

Regina Haars, Ph.D.

Horst Hinsen, Ph.D.

Jane Houldsworth, Ph.D.

Stephen W. Hunt III, Ph.D.

James B. Hurley, Ph.D.

Huey Ru Hwu, Ph.D.

Akira Ishihara, Ph.D.

Lianna Johnson, Ph.D.

Reid C. Johnson, Ph.D.

Robert J. Kaiser Jr., Ph.D.

Karen S. Katula, Ph.D.

Christine Kinnon, Ph.D.

Joan A. Kobori, Ph.D.

Mitchell Kronenberg, Ph.D.

Chia-Lam Ku, Ph.D.

Eric H. C. Lai, Ph.D.

Ulf Landegren, Ph.D.

Ren-Jang Lin, Ph.D.

James P. Lugo, Ph.D.

Daniel Margoliash, Ph.D.

Paolo Mariottini, Ph.D.

Ryn Miake-Lye, Ph.D.

Kathleen L. McGuire, Ph.D.

Andrew P. McMahon, Ph.D.

Randall T. Moon, Ph.D.

Norihiro Mutoh, Ph.D.

Nalini Narashiman, Ph.D.

Kenji Oosawa, Ph.D.

Harry S. Orbach, Ph.D.

Irene V. Pech, Ph.D.

Eric M. Phizicky, Ph.D.
## Research Fellows (continued)

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<td>Ronald H. A. Plasterk</td>
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<td>Cesare Rossi</td>
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<td>Minnie McMillan</td>
<td>University of Southern California</td>
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<td>Carol A. Miller</td>
<td>USC School of Medicine</td>
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<td>Julio Montoya</td>
<td>Universidad de Zaragoza, Zaragoza, Spain</td>
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<td>Polly Henninger Pechstedt</td>
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<td>Birgit Satir</td>
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<td>Evelyn T. Teng</td>
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<td>Ontario Cancer Institute, Toronto</td>
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<td>Jae Sub Yang</td>
<td>Daegu University, Seoul, Korea</td>
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<td>Jenny Yates-Hammett</td>
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<td>University of California, Los Angeles</td>
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<td>James J. Lee</td>
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<td>Donna L. Livant</td>
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<td>Michael A. Lochrie</td>
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## Visiting Associates

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<tr>
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<tr>
<td>L. Elizabeth Bertani</td>
<td>Karolinska Institute, Stockholm</td>
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<tr>
<td>Edward N. Brody</td>
<td>Institut de Biologie-Physico-Chemique, Paris</td>
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<tr>
<td>R. Andrew Cameron</td>
<td>University of Puerto Rico</td>
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<tr>
<td>Palmiro Cantatore</td>
<td>Instituto di Chimica Biologica, University of Bari, Italy</td>
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<tr>
<td>Lynn Daigarno</td>
<td>Australian National University</td>
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<tr>
<td>Rolf Dermietzel</td>
<td>University of Essen, West Germany</td>
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<tr>
<td>Bert Ely</td>
<td>University of South Carolina</td>
</tr>
<tr>
<td>Elizabeth Grayhack</td>
<td>University of California, San Francisco</td>
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<td>David R. Hinton</td>
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<td>Steven A. Johnson</td>
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<td>Byung S. Kim</td>
<td>Northwestern University Medical and Dental Schools, Chicago</td>
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<td>Joan L. Klotz</td>
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<td>Honghua Li</td>
<td>Shandong College of Agriculture, The People's Republic of China</td>
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<td>Peter Lonai</td>
<td>Weizmann Institute, Rehovot, Israel</td>
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<td>Bernard Malissen</td>
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<td>Tim Hunkapiller</td>
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<td>Michael A. Lochrie</td>
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## Graduate Students

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<td>Elliot Altman</td>
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<td>Lois M. Banta</td>
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<td>Mark K. Bennett</td>
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<td>Beverley J. Bond</td>
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<td>Edward Callaway</td>
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<td>Caren Chang</td>
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<td>Michael R. Emerling</td>
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<td>Douglas A. Fisher</td>
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<td>Boning Gao</td>
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<td>Mark D. Garfinkel</td>
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<td>Michael A. Lochrie</td>
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Summary: Our laboratory has been interested in the mechanisms of control of gene expression. In recent years, we have concentrated on the study of RNA splicing in yeast. All three classes of eukaryotic genes contain introns: tRNA genes, rRNA genes, and genes coding for nuclear mRNAs. In each case, the intron(s) is removed from an RNA precursor by RNA splicing. Research from a number of laboratories has shown that splicing of each of the three classes of precursors proceeds by different mechanisms. tRNA splicing is mediated by enzymes and requires ATP, while rRNA splicing, as studied by Cech and his colleagues (Kruger et al., 1982), does not require protein but instead is self-catalyzed. mRNA splicing requires ATP and both protein and RNA components, but less is known about the mechanism of this reaction.

We have continued to study the mechanism of tRNA splicing in yeast. Two components are required for this reaction, an endonuclease and a ligase. The endonuclease is apparently an integral membrane protein. It is present in low quantities in yeast and has been extremely difficult to purify. Good progress has been made this year, however, and we are optimistic that pure enzyme can be obtained soon.

The ligase protein has been purified to homogeneity. It contains three activities, a phosphodiesterase, a polynucleotide kinase and is an RNA ligase in a single 90 kD polypeptide chain. The gene for this protein has been cloned. Upstream and overlapping the ligase gene by 8 bp is an open reading frame coding for a potential protein of 122 amino acids. This protein, as judged from its hydropathy plot, would be an integral membrane protein. We are eager to find out whether this gene is expressed, whether it is expressed coordinately with ligase and what the function of the gene product may be. Could this be the gene for the endonuclease?

Using the pure ligase protein as antigen we have prepared rabbit anti-ligase and this antibody has been used to investigate the location of ligase in the yeast cell. Indirect immunofluorescence studies localized the ligase to the nucleus and immunoelectron microscopy indicates that a primary location is the nuclear membrane. If the endonuclease is an integral nuclear membrane protein, it could provide the target for ligase localization in the cell. Studies on the basis of substrate recognition of the tRNA precursor have continued. A series of experiments were performed with precursors derived from a tRNA_{_Leu}^1 gene containing a synthetic intron. These experiments prove that the structure of the intron at the 3' splice junction is crucial. These studies can now be extended with a totally synthetic tRNA_{_Phe}^1 gene linked to a bacteriophage T7 promoter. With this system, synthetic precursors can be prepared at will and variations of both the intron and the mature domain can be studied.

We will continue with our study of yeast tRNA splicing at three levels. 1) We wish to learn more about the detailed mechanisms of both of the enzymatic reactions and the substrate requirements for each. 2) We will initiate a genetic study of the ligase and eventually the endonuclease. 3) In an extension of the cytology that has already been done, we wish to understand the cellular location of these enzymes and their role (if any) in the transport of tRNA from nucleus to cytoplasm.

In the past year, we have intensified our study of yeast mRNA splicing. An in vitro system has been developed and studies on the effects of intron mutants on the in vitro splicing pathway have been carried...
out. We are just beginning to fractionate the system and enumerate the components.

Success in obtaining in vitro mRNA splicing came as a result of the development of an assay that was both dependable and very sensitive. The assay utilized an oligonucleotide that paired with mRNA at the splice site allowing protection from digestion by S1 nuclease. Using this assay, mRNA splicing was detected in yeast whole cell extracts and optimal conditions for in vitro splicing were determined.

The reaction appears to take place in two stages. First, the 5' splice junction is cut and coordinately the 5' nucleotide of the intron is covalently joined to the last A in the conserved TACTAAC sequence near the 3' end of the intron and the 3' exon. The 3' splice junction is cleaved and joined to the 5' splice junction. This reaction scheme is identical to that seen in mammalian extracts (Grabowski et al., 1984; Krainer et al., 1984; Padgett et al., 1984; Ruskin et al., 1984).

We have constructed several alterations in the precursor RNA by oligonucleotide-directed mutagenesis. These alterations affect mRNA splicing both in vivo and in vitro and have provided some insight into the role of conserved intron sequences. The mechanism by which the 5' and 3' splice junctions interact in mRNA splicing remains a mystery. Base pairing between 5' and 3' splice junctions does not appear to provide sufficient specificity so other cellular components, particularly small nuclear ribonucleoprotein particles, have been hypothesized to provide that specificity. Evidence for their participation in the splicing reaction in mammalian cells has been provided by other laboratories (Mount et al., 1983; Padgett et al., 1983; Kramer et al., 1984). We have discovered that mRNA splicing in yeast takes place on a 40S particle that we have called the "spliceosome." Presumably, this particle contains factors that fold the precursor into a configuration allowing precise splicing to take place. We are currently trying to determine the composition of the spliceosome and in particular whether it contains small nuclear RNAs.

We have continued with our study of synthetic tRNA genes. This work now has two aspects. To understand the basis for tRNA synthetase recognition of tRNA, we have attempted to change the identity of a tRNA\textsuperscript{Leu} to tRNA\textsuperscript{Ser}. As reported last year, this transformation was successfully accomplished. We have further proved this by using the altered tRNA\textsuperscript{Leu} amber suppressor to suppress an amber mutation at position 10 of the \textit{E. coli} dihydrofolate reductase gene. The suppressed protein was purified and sequencing proved that it contained a serine residue at position 10. Now, we must precisely define which of the 12 base changes in tRNA\textsuperscript{Leu}\textsuperscript{Ser} are essential for specificity.

In collaboration with Jeffrey Miller’s laboratory at UCLA, we have undertaken a project to create new \textit{E. coli} amber suppressors. The amber suppressor genes are synthesized \textit{de novo} as in the case of the tRNA\textsuperscript{Leu} suppressor and given the amber anticodon, CTA. So far, we have synthesized suppressors specific for the amino acids Phe, Cys, Arg, His, Lys and Glu. These suppressors range in efficiency from 10% to 80%. This expands the number of amino acids that can be inserted at any amber site from five to 11, offering interesting prospects for a genetic attack on problems of protein structure and function.

References:


1. \textit{In vivo} Role of tRNA Ligase Protein

Eric M. Phizicky

\textit{Saccharomyces cerevisiae} tRNA ligase protein is one of the components of the tRNA splicing apparatus in the yeast cell. Several yeast tRNA genes contain intervening sequences that, when transcribed, must be removed by splicing in order to generate mature tRNA. Splicing of the precursor tRNA is effected in two steps: an endonuclease cuts the precursor molecule once on either side of the intervening
sequence; and tRNA ligase splices together the two remaining tRNA half-molecules.

We are interested in the in vivo role of the tRNA ligase protein. With what other cellular proteins does it interact and in what other cellular processes (such as tRNA transport or other splicing events) does tRNA ligase protein play a role? To this end, we have purified the protein to near homogeneity and isolated the structural gene. We have constructed yeast and E. coli strains in which tRNA ligase protein is overproduced. With large amounts of the protein, we hope to be able to identify interacting proteins. We also have shown that disruption of the tRNA ligase gene is lethal to the cell, and we are in the process of constructing conditional mutants in the gene in order to study the physiological role of the protein in the cell.

2. \textit{Saccharomyces cerevisiae} tRNA Ligase Gene

\textbf{Shawn Westaway, Eric M. Phizicky}

As previously reported, the gene for yeast tRNA ligase has been cloned by Eric Phizicky. The 4 kb fragment containing the gene was subcloned into five fragments and inserted in each orientation in M13 cloning vectors for the purpose of sequencing by the Sanger dideoxy method, using oligonucleotides as sequencing primers (provided by Dr. S. Horvath and Ms. M. Tomich). During the course of sequencing the gene and its flanking regions, an open reading frame (ORF) was discovered 5' to the initiation codon of tRNA ligase. This ORF is 122 amino acids long, has a start codon and occurs on the same strand as ligase, but is out-of-frame. The stop codon of this ORF lies 8 bp downstream of the start codon of tRNA ligase. We are in the process of determining if this ORF codes for RNA \textit{in vivo}. The C terminus of the ORF resembles transmembrane domains of known integral membrane proteins with respect to hydrophobicity profiles. If the ORF is transcribed, it will be interesting to characterize the RNA and to determine if it codes for a protein. It has been previously reported that partially purified yeast tRNA ligase protein contains several separate activities associated with tRNA splicing, suggesting that the protein is composed of separate active sites (Greer et al., 1983). It will be interesting to use \textit{in vitro} mutagenesis to locate these active domains and to create mutant strains in yeast.

defective in one or more aspects of yeast tRNA splicing.

\textbf{Reference:}


3. Highly Purified tRNA Splicing Endonuclease from \textit{Saccharomyces cerevisiae}

\textbf{Phillip R. Green}

The tRNA splicing endonuclease and ligase enzymes catalyze the two-step reaction necessary for the maturation of the few precursor tRNAs of yeast that contain intervening sequences (Peebles et al., 1979). Whereas the ligase has been purified to homogeneity and its structural gene has been cloned, the membrane-bound endonuclease (Peebles et al., 1983) has resisted purification. To understand in detail the physiological role of these enzymes and the splicing reaction, the endonuclease must be purified.

The following procedure has enabled us to obtain small quantities of highly purified endonuclease. Cells were disrupted using a Dyno-Mill in buffer containing 1.0 M ammonium sulfate. Crude membranes were isolated following successive low and high speed spins. They were subjected to a series of washes in buffer containing high salt, low salt plus low detergent, and high detergent without salt. Activity was extracted from the membrane with 0.9% Triton X-100 plus 0.1 M ammonium sulfate. The extract was chromatographed over heparin agarose, flowed through DEAE-Sephacel, bound to single-stranded DNA cellulose and eluted with salt plus yeast tRNA in 1.0% Triton X-114. Many contaminating proteins and tRNA were removed by phase separation. Sodium dodecyl sulfate gel electrophoresis of the final fractions exhibited two major polypeptides plus minor contaminants. However, the yield of activity is very low and we have been attempting to determine whether either of the remaining major peptides is the endonuclease. Work on improving the purification procedure and on the identification of the endonuclease polypeptide is progressing.

\textbf{References:}


4. The Cellular Location of tRNA Ligase in Saccharomyces cerevisiae

Michael W. Clark

Some of the yeast tRNA genes contain intervening sequences (IVS). The removal of the IVS from the precursor tRNA is one of the last steps in tRNA maturation before the tRNA is transported out of the nucleus. The mechanism that removes the IVS from the yeast precursor tRNAs, referred to as the tRNA splicing mechanism, has at least two distinct steps—an ATP-independent endonuclease step that removes the IVS, and an ATP-dependent step that ligates the two resulting tRNA halves, the tRNA ligase. The endonuclease activity has the characteristic of an integral membrane protein, requiring a detergent extraction of the cell membranes. While the tRNA ligase acts as a peripheral membrane protein, it requires a high salt extraction at the initial step of purification (see the abstracts by Green and Phizicky for further information on the purification of these proteins). How these two activities interact during the splicing process and what role, if any, these activities have in tRNA transport are not understood. We are examining these components of the tRNA splicing process at a cellular level by immunocytological techniques, attempting to determine the exact cellular location of these splicing events. With the splicing components localized during active cell growth, we might be able to infer, at least at a gross level, how the components interact during the splicing process. At this time, we have an antibody directed against the tRNA ligase protein. This antibody has been utilized at both an optical and electron microscopic level to determine the cellular location of the tRNA ligase.

Indirect immunofluorescence on wild-type yeast cells has localized the tRNA ligase in the cell nucleus. Similar studies with a strain of yeast that contains a Cen 3 plasmid containing the tRNA ligase gene under galactose-inducible Gal 10 promoter control have shown that after galactose induction there is an accumulation of the tRNA ligase occurring in the yeast nucleus. Indirect immune electron microscope studies on wild-type yeast have mapped the tRNA ligase at two sites in the nucleus—one on the nuclear envelope, predominantly the inner membrane, and a second site in the nucleoplasm about 150-350 Å from the inner nuclear membrane. Immune electron microscope studies on the galactose-inducible tRNA ligase strain of yeast show a dramatic increase in the nucleoplasmic tRNA ligase concentrations, while maintaining an almost identical level of nuclear envelope staining as seen in wild-type cells. Further electron microscope studies are under way following the induced tRNA ligase to examine the pathway of the protein into and maybe out of the nucleus. When antibodies against the endonuclease are available, a similar mapping procedure will be done for this protein.

5. Effect of Intron Mutations on Splicing of S. cerevisiae SUP53 Pre-tRNA

Marjorie C. Strobel-Fidler

We are studying the role of the intervening sequence in the Saccharomyces cerevisiae leucine-inserting, amber suppressor gene SUP53 (a tRNA^Leu^ allele). Previously, we showed that the totally intact intron is necessary for proper base modification of the precursor tRNA in vitro and, in turn, full suppressor function of the mature tRNA in vivo. Additional SUP53 mutants were constructed to investigate the role of intron sequence and structure on recognition of the precursor by the S. cerevisiae tRNA endonuclease and ligase.

Two types of mutant intron were introduced: one that represents an internal deletion of the natural SUP53 32-base intron and one that introduces a novel intron. In each case, additional alterations were made in splice junction proximal nucleotides. The mutant genes were transcribed and end-processed in vitro using a yeast nuclear extract developed by Prof. Carl Parker (Division of Chemistry and Chemical Engineering, Caltech). All mutants were efficiently transcribed and end-matured. The end-trimmed transcripts were assayed for splicing in vitro using partially purified S. cerevisiae tRNA endonuclease and ligase.

While many of these intron mutants are effectively spliced, certain mutants are refractory to tRNA endonuclease cleavage. The cleavage defects are partially rescued by additional base changes in the intron. In all cases, the RNA processing pattern in vitro correlates with the mature tRNA function in vivo, as assayed by suppressor tRNA function.

Previous genetic studies have shown that mutations
that affect splicing and end-processing map to a precursor tRNA's mature domain. However, these intron-mutated precursors do not perturb the mature domain. Rather, the computer-derived secondary structures indicate that only the intron structure of these mutants may be significantly altered. The intron structures provide information on the features of a precursor tRNA that are critical for tRNA endonuclease activity.

6. A Novel Approach to the Study of tRNA Splicing

Vicente M. Reyes, Marjorie C. Strobel-Fidler

tRNA splicing in Saccharomyces cerevisiae is mediated by an endonuclease and an RNA ligase (Greer et al., 1983; Peebles et al., 1983). However, not enough is presently known about the substrate specificities of these splicing enzymes.

Genetic studies have shown that mutations that affect processing largely map to the conserved features of the mature domain of pre-tRNA. These changes either affect the internal tRNA gene promoters or some structural feature critical for end maturation. Consequently, it has been difficult to obtain sufficient quantities of a precursor tRNA in vitro and hence to study the effects of these mutations on splicing. Further, there is presently no direct information on the higher-order structure of a pre-tRNA molecule.

To circumvent these problems, a yeast pre-tRNA^Phe gene under the control of a strong bacteriophage T7 promoter has been constructed from synthetic oligodeoxynucleotides. Using the T7-specific RNA polymerase, large quantities of the tRNA^Phe precursor have been made.

The precursor tRNA made from this gene is accurately spliced in vitro by the purified S. cerevisiae enzymes, immediately suggesting that post-transcriptional base modifications are not necessary for the activity of these enzymes.

Experiments are under way to define the substrate specificities of the yeast splicing machinery at the primary, secondary, and tertiary levels of structure by introducing mutations in the anticodon-loop-intron region of the pre-tRNA, and also those that disrupt the tertiary base interactions that stabilize the overall "L" configuration of the molecule.

References:

7. Coordinate Expression of a tRNA Gene Tandem in Yeast

Vicente M. Reyes, Andrew J. Newman

It is known that tRNA genes occur in the yeast genome as highly dispersed, independent monocistronic transcriptional units. The discovery of the 5'-tRNA^Arg-tRNA^Asp-3' gene tandem, separated only by a 10 base pair (bp) spacer sequence, thus represents an extremely rare case of tight clustering of these genes. Previous work in this laboratory (Kjeilin-Straby et al., 1984) indicated that transcription of this gene tandem in vitro by a polIII transcription system derived from a crude yeast extract (C. Parker and J. Topol, unpublished results) is solely governed by the intragenic promoters of the upstream tRNA^Arg gene: a dimeric tRNA precursor is produced containing the two gene sequences, the spacer sequence, and short leader and trailer sequences. This dimeric precursor is then processed in the extract into mature tRNA^Arg and tRNA^Asp molecules (Engelke et al., 1985).

Given that both the tRNA^Arg and tRNA^Asp genes in this tandem contain the requisite consensus internal promoter sequences necessary for transcription, the question remains as to why the tRNA^Asp gene fails to direct its own transcription.

We have shown that mutation (C66G) or deletion (ΔArg) of the tRNA^Arg gene fails to switch on independent transcription of the tRNA^Asp gene. However, deletion of both the tRNA^Arg gene and the spacer allows independent tRNA^Asp transcription. This means that the 10 bp spacer is inhibitory to tRNA^Asp transcription.

How does the spacer sequence prevent tRNA^Asp transcription? Transcription of tRNA genes in yeast generally starts at a unique A residue in a short string of As 8-12 bases upstream of the gene. The spacer sequence does not contain A. Thus, a string of 3 As was introduced at the 5' terminus of the spacer sequence in ΔArg. This mutant (A3ΔArg) does give rise to transcription of the tRNA^Asp gene, indicating that the lack of a polIII start site in the spacer sequence prevents tRNA^Asp transcription in ΔArg. However,
when the same string of As is introduced into the spacer sequence in C56G, the resulting double-mutant (A3C56G) is phenotypically identical to C56G. No independent trNA\textsubscript{Asp} transcription is found. This unexpected result seems to suggest that an upstream trNA gene in close proximity to a second may also be inhibitory to the independent transcription of the latter.

References:

8. Yeast mRNA Splicing in vitro
Ren-Jang Lin, Andrew J. Newman, Sao-Chen Chang, Gloria Dalbadie-McFarland, Usha Vijayraghavan

Yeast actin and CYH2 mRNA precursors (pre-mRNA) containing a single intron can be synthesized in an SP6 in vitro transcription system. These synthesized pre-mRNAs are accurately spliced in a soluble whole-cell extract of yeast. A very sensitive oligonucleotide/nuclease S1 assay was developed to detect the appearance of mRNA in the in vitro splicing reaction. Splicing in vitro requires ATP, and non-hydrolyzable ATP analogs cannot substitute for ATP. No apparent time lag was observed. The products of the splicing reaction were isolated and characterized using \textsuperscript{32}P-labeled synthetic pre-mRNA as substrates in the in vitro splicing system. The excised intron is released as a lariat in which an RNA branch connects the 5' end of the molecule to the last A in the "intron conserved sequence" UACUAAC. Two other discrete RNA species produced during splicing in vitro may represent reaction intermediates: free, linear exon 1 and a form of the intron lariat extending beyond the 3' splice site to include exon 2. Both lariat forms correspond to molecules previously shown to be produced during yeast pre-mRNA splicing in vivo. These results suggest that mRNA splicing in yeast shares extensive similarities with the mammalian process.

Two point mutations in the intron of the yeast CYH2 gene were constructed, and their effects on CYH2 pre-mRNA splicing were analyzed in vivo and in vitro. The first mutation prevents splicing in vivo. Furthermore, synthetic CYH2 pre-mRNA carrying the TACTACG alteration is not specifically cut or rearranged in any way in a yeast mRNA splicing system. The second mutation, which alters the first G of the intron to A, converting the highly conserved GTATGT 5' splice site sequence to ATATGT, also blocks intron excision in vivo and in vitro, but in a radically different way. Pre-mRNA carrying this mutation was still cut normally at the mutant 5' splice site in vitro, to give authentic exon 1 and an intron-exon 2 lariat RNA with an A-A 2'-5' phosphodiester linkage at the branch point. This intron-exon 2 lariat RNA is a dead-end product. We conclude that the subsequent cleavage at the 3' splice site must be sensitive to the sequence of the 5' end of the intron attached at the branch point.

9. Characterization of Mutants in Actin Intron Sequences
Usha Vijayraghavan

Our experimental approach in understanding the molecular mechanism of mRNA splicing in yeast has been to make specific base substitutions in the conserved splicing signals found in yeast introns. We have used the yeast actin gene as the template for oligonucleotide site-directed mutagenesis. To assess the influence in vitro of these mutations on pre-mRNA recognition by the splicing apparatus and to separate the splicing process into discrete steps, we have used the SP6 transcription system and the in vitro splicing system developed in our laboratory.

Two different point mutations were made in the 5' splice site. Changing the conserved GTATGT to ATATGT or CTATGT abolishes splicing. This indicates that the first base of the actin intron plays an important role in the recognition of the 5' splice site for the first step of the splicing reaction and no cryptic 5' splice sites are activated when this base is altered. On the other hand, alteration of the normally conserved G at the last position of exon 1 to C has no effect on splicing and splicing proceeds to completion.

The consequences of mutation in the internal TACTAAC found in yeast introns was also analyzed. When the branch point in lariat RNAs (the last A in the TACTAAC sequence) is altered to a C, the mutant pre-mRNA is not spliced in vitro. In the absence of a suitable branch point, no cleavage at the 3' splice site is seen. A mutation changing the second T of this sequence to an A also results in an inactive substrate.
Hence, recognition of an acceptable TACTAAC sequence seems crucial for cleavage at a normal 5' splice site.

Lastly, a mutation in the 3' splice site from a PyAG to a PyAC allows a formation of lariat IV-exon 2 and excised exon 1, but these are dead-end products and no downstream PyAG is utilized for the second step of the reaction. This suggests that the presence of a 3' splice site at an acceptable distance from the branch point is needed for cleavage at this site.

10. Fractionation and Characterization of mRNA Splicing Components in Yeast

Soo-Chen Cheng

We have fractionated a yeast whole cell extract that can accurately splice synthetic actin and CYH2 pre-mRNAs. Three components required for splicing have been separated by use of ammonium sulfate fractionation and chromatography on phosphocellulose column. These components are designated I, II, and III. Each component alone has no splicing activity. Addition of components I and II to the reaction mixture gives the first step of splicing reaction, producing only splicing intermediates. Addition of component III completes the reaction.

Components I and II, but not III, are sensitive to micrococcal nuclease, indicating some RNA moiety is associated with these two components, but not protected by the proteins from nuclease digestion. Experiments using antiserum against (m3G)CAP, a typical structure of snRNA, give the following results. 1) Pre-treating the crude extract with the antiserum followed by removing the antiserum gives an inactive splicing extract, indicating some component(s) essential for splicing is depleted from the extract. 2) Incubating the splicing reaction with antiserum pre-absorbed to protein A-Sepharose precipitates RNA substrates, the precursor as well as the spliced products except the message, indicating at least one RNA moiety is associated with a large splicing complex.

These results implicate that as in the mammalian system, snRNPs are involved in the splicing reaction. Components I and II are likely to contain snRNPs.

II. The Yeast "Spliceosome"

Edward N. Brody

The process of pre-messenger RNA (pre-mRNA) splicing is central to the understanding of eukaryotic gene expression. Pre-mRNA contains sequences that code for protein (exons), but this information is often interrupted by one or more sequences (introns) that must be removed to allow formation of a contiguous messenger RNA (mRNA) molecule. Splicing consists of removing intron RNA and rejoicing appropriate exon RNAs into such a contiguous mRNA. This cutting and rejoicing poses a number of problems, which are not yet resolved. Introns can be very large, yet the two exonic RNA sequences must be brought into proximity in order to be spliced. Also, the splicing must be precise (and rapid). Once cut, a 5' exon RNA molecule (exon 1 RNA), must join with the appropriate 3' exon (exon 2 RNA) and with no other. Clearly, binding pre-mRNA into a supramolecular complex and holding cut exon RNA intermediates in such a complex would be one way to facilitate accurate splicing.

The splicing of pre-mRNA in Saccharomyces cerevisiae, as in HeLa cell extracts, takes place via two apparently concerted reactions (Figure 1). In a first step, pre-mRNA is cut at the exon 1-intron junction with simultaneous formation of a lariat RNA form containing the intron (IVS) and exon 2 (IVS-exon 2); we have previously referred to this as the "2/3 molecule"). The lariat in this reaction product is the consequence of a 2'-5' phosphodiester bond formed between the 2' OH group of the last A in the conserved UACUAAC (TACTAAC box) sequence and the pG of the 5' end of the intron (Figure 1; A, adenine; C, cytosine; T, thymine; G, guanine, U, uracil). In a second step, IVS-exon 2 is cut at the intron-exon 2 junction, and exon 1 and exon 2 are ligated to give mRNA. This endonucleolytic cut releases intron RNA in a lariat form (Figure 1). In yeast whole cell extracts, these reactions can be carried out with fidelity.

The in vitro splicing reactions of pre-messenger RNA in a yeast whole cell extract were analyzed by glycerol gradient centrifugation. Labeled pre-messenger RNA appears in a 40S peak only if the pre-messenger RNA undergoes the first of the two partial splicing reactions. RNA analysis after extraction of glycerol gradient fractions shows that lariat-form
intermediates, molecules unique to messenger RNA splicing, are found almost exclusively in this 40S complex. Another reaction intermediate, cut 5' exon RNA, can also be found concentrated in this complex. The complex is stable even in 400 mM KCl, although at this salt concentration, it sediments at 35S and is clearly distinguishable from 40S ribosomal subunits. This complex, termed a "spliceosome," is thought to contain components necessary for mRNA splicing; its existence can explain how separated exons on pre-mRNA are brought into contact.

References:

![Figure 1. The splicing pathway of pre-mRNA. The reactions are described in the text. The intron-exon junctions are represented by vertical bars. This pathway of two apparently concerted reactions is found in S. cerevisiae, both in vivo and in vitro, as well as in extracts prepared from HeLa cells.](image)

12. Study of the Functional Components of the Yeast Spliceosome

Gloria Dalbadie-McFarland

mRNA splicing in yeast proceeds via a two-step reaction carried out by a multicomponent system. This splicing complex consists of at least three components that can be fractionated by ammonium sulfate precipitation. They have been designated components I, II and III (S.-C. Cheng, unpublished; see Abstract No. 10). During in vitro splicing of actin and CYH pre-mRNA by yeast crude whole cell extracts, both the RNA precursor and splicing reaction intermediates have been found to sediment in glycerol gradients at 40S, presumably due to association with the cell's splicing apparatus (Brody and Abelson, 1985).

The objective of the present study is to determine the role of each of the fractionated components in the formation of the 40S complex, which has been named "spliceosome" by Brody and Abelson (1985). To this effect, pre-mRNA binding to components I, II and III has been analyzed by glycerol gradient centrifugation.

Components I plus II form a 40S particle when incubated with labeled wild-type mRNA precursors that have all the required splicing signals. In the absence of ATP, which is absolutely required for splicing to occur, pre-mRNA incubated with components I and II peaks at approximately 25S in a glycerol gradient, which resolves this from free RNA. It appears, then, that pre-mRNA is recognized by a part of the splicing apparatus that associates with additional components to form a 40S particle in an ATP-dependent step. Experiments to test this hypothesis are in progress.

Using mutant actin mRNA precursors (U. Vijayaraghavan, unpublished; see Abstract No. 9), it has been determined that both the conserved 5' splice site sequence (GTATGT) and the conserved TACTAAC sequence in the intron are involved in recognition of the RNA and formation of the 40S complex by components I and II. The 3' splice junction does not appear to be involved in complex formation.

Preliminary results indicate that neither component I nor component II alone is capable of forming the 40S complex with labeled pre-mRNA. In glycerol gradients, other species that migrate more slowly than the 40S complex species appear when component I, free of component II activity, is
alkaline extraction of nuclei, RNA digestion occurs in association of the pre-mRNA with any large particle containing components believed that a major component of the putative "spliceosome" is hnRNP. In higher eucaryotes, hnRNP splicing, and transport from the nucleus. It could be cation of the major hnRNP protein in yeast. Extraction consists of a series of transcripts undergo the subsequent steps of processing, between particles, releasing them as RNA fibril (''beads-on-a-string'' structure). Brody, E. and Abelson, J. (1985) Science 228, 963-967.

13. Purification of hnRNP Proteins from Yeast and their Role in mRNA Splicing

Michael E. Cusick

Immediately after transcription, precursor mRNAs in the cell nucleus are packaged with a simple set of proteins of 30-40,000 dalton molecular weight (MW) to form the complex known as heterogenous nuclear ribonucleoprotein (hnRNP). As hnRNP, the nascent transcripts undergo the subsequent steps of processing, splicing, and transport from the nucleus. It could be believed that a major component of the putative "spliceosome" is hnRNP. In higher eucaryotes, hnRNP consists of a series of 20-25 nm particles along the RNA fibril ("beads-on-a-string" structure). Upon mild alkaline extraction of nuclei, RNA digestion occurs between particles, releasing them as 30-40S subunits. The structure of hnRNP in yeast is not known.

I have undertaken the identification and purification of the major hnRNP protein in yeast. Extraction of yeast nuclei yields a 30S RNP whose major protein is 30,000 daltons in MW. This protein was partially purified from a crude yeast extract by its passage through double-stranded DNA cellulose but its retention on single-stranded DNA cellulose. Further purification on heptyl agarose yields a fraction about 25% pure for the 30K protein. This fraction binds to single-stranded mRNA transcripts to make complexes that sediment at 20S and show 15-20 nm beads by electron microscopy. The 30K protein, after isolation by preparative SDS-PAGE, has an amino acid composition similar to hnRNP proteins from higher organisms.

Upon complete purification of the 30K protein, I will partially sequence it in order to construct synthetic DNA oligonucleotides to use as probes for the cloning of its gene. Also, polyclonal antibodies to the 30K protein will be raised. The lab can then use these tools in conjunction with the in vitro mRNA splicing system to decipher the role of hnRNP in splicing and to find any hnRNP protein(s) actively involved in splicing.

14. Changing the Identity of a tRNA Molecule

Jennifer Normanly, Suzanna J. Horvath, Richard C. Ogden

Aminoacyl-tRNA synthetase catalyzes the aminocylolation of tRNA, resulting in an ester linkage between the 3' end of the tRNA and the amino acid. The recognition of a tRNA by its cognate synthetase has been defined only in general terms; the features of a tRNA, which result in its exclusive recognition by a specific synthetase, remain a mystery.

Our approach to further elucidate the specific nature of the synthetase-tRNA recognition is to convert a leucine-inserting amber suppressor tRNA into a serine-inserting amber suppressor tRNA. Using synthetic oligonucleotides, we have constructed the gene for E. coli leucine-inserting amber suppressor tRNA (tRNALeu) as well as a mutant (tRNALeu+Ser) which contains 12 base changes. The alterations were made in positions presumed to be regions of contact between the tRNA and synthetase. To screen for the insertion of serine into amber (non-sense) codons, we constructed an amber mutation in the active site serine codon of the β-lactamase gene. We have shown in vivo that this amber mutation is suppressed by the insertion of serine but not leucine. The synthetic tRNALeu+Ser gene produces a functional suppressor tRNA as evidenced by its ability to suppress amber mutations in the lac operon. The tRNALeu+Ser gene will suppress the amber mutation in β-lactamase as well as in the lac operon, albeit at a very low efficiency (0.5-1.0%) in comparison to the tRNALeu (60-90%).

Recently, we have developed a method to determine the exact specificity of the tRNALeu+Ser. The mutant tRNA was utilized to suppress an amber mutation in the tenth residue of E. coli dihydrofolate reductase (DHFR) gene. After purification by affinity chromatography, the mutant DHFR was subjected to N-terminal sequence analysis. This assay reveals that the tRNALeu+Ser inserts serine in response to amber codons. We are currently working to increase the suppression efficiency of the tRNALeu+Ser.

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15. Construction and Use of an E. coli Amber Suppressor tRNA Bank

Jennifer Normanly, John N. Abelson, Lynn C. Kleina\(^1\), Jean-Michel Masson\(^1\), Jeffrey H. Miller\(^1\)

We have used gene synthesis in vitro to begin constructing a series of E. coli strains carrying amber suppressors which can insert different amino acids in response to the amber (UAG) codon. Both strands of each respective tRNA gene are synthesized as a series of six oligonucleotides of approximately 30 base pairs (bp) in length, which are annealed and ligated in vitro, and then inserted into a plasmid cloning vehicle under control of either the lac or tpp promoter. Each tRNA gene contains the identical sequence as the wild-type tRNA, except for the anticodon, which has been altered to read UAG. We have constructed high efficiency suppressors derived from tRNAs that insert histidine, arginine, lysine, cysteine and phenylalanine, and low efficiency suppressors derived from tRNAs that insert aspartic acid, threonine, and proline. In order to determine the absolute specificity of these novel tRNAs, they are being utilized to suppress a nonsense mutation in the tenth residue of the E. coli dihydrofolate reductase (DHFR) gene. After purification by affinity chromatography, the mutant DHFR will be subjected to N-terminal sequence analysis. Using this assay, we have verified that the suppressors derived from phenylalanine and cysteine tRNAs insert the expected amino acid.

We are currently synthesizing additional amber suppressors and are also examining the effect of a large series of amino acid substitutions in specific proteins. The lac repressor is one object of our study, since Jeffrey Miller has characterized about 100 amber mutations at known positions in the gene and corresponding protein. We have monitored the effects of about 800 amino acid substitutions in the repressor by employing all of the existing suppressors including those in this study.

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16. Characterization of a Yeast Regulator (a1 Protein) of Cell Type-specific Gene Expression

Elizabeth Grayhack

The yeast Saccharomyces cerevisiae has three distinct cell types: a (haploid), A (haploid), and a/a (diploid). The cell type is determined by a single genetic locus, the MAT locus, but it is exhibited in multiple cell type-specific phenotypes as a result of differential gene expression. The MAT allele codes for the two proteins, a1 and a2, which are hypothesized to turn on expression of the a-specific genes and to turn off expression of the a-specific genes, respectively. To understand how the a1 gene product acts on the expression of a-specific genes, we are attempting to purify the a1 protein and examine its activity in vitro.

To obtain a rich source of a1 protein, we fused the a1 gene to a strong inducible yeast promoter element. The a1 protein produced from this gene is apparently active because an a-specific gene is fully induced in yeast cells carrying this a1 gene. Under inducing conditions, yeast cells carrying this clone produce greater than 100 fold more a1 message than a wild-type cell, suggesting that the a1 protein is likely to be enriched in cells carrying this plasmid.

In the absence of a yeast transcription system, in which the activity of the a1 protein (provoking expression of an a-specific gene) could be measured, we are developing an assay for the polypeptide product of the a1 gene. Purified a1 message selected by hybridization is translated in a yeast extract, yielding radiochemically labelled a1 polypeptide that is approximately 5% radiochemically pure. We intend to add this polypeptide to yeast extracts and examine purification of the labelled polypeptide looking for copurification of an unlabelled protein from the a1 over-producer strain. With purified a1 protein, we will ask if a1 protein binds directly to DNA near the a-specific genes, if a1 protein provokes the binding of other proteins to DNA, and if a1 protein interacts with other yeast proteins. Eventually, we hope to understand how a1 protein turns on expression of a-specific genes.

17. Cloning of the cDNA for the Globin from the Clam, Barbatia reeveana

Austen F. Riggs, Clare Riggs, Ren-Jang Lin, Horst Domdey\(^1\)

Grinch and Terwillinger (1980) discovered that the clam Barbatia reeveana has two intracellular hemoglobin, one with 12-14 polypeptides of 33 kilodaltons (kd) each and the other with two kinds of 16-17 kd chains forming a tetramer. Red cells were isolated from the clams, washed and transported on liquid nitrogen by air from Baja, California. RNA from these
cells was active in a rabbit reticulocyte system. Poly-
acrylamide gel electrophoresis showed that the label
(35S-methionine) was incorporated primarily into a
single major 32-35 kd band and two minor bands of 14
kd and 16 kd, exactly what was hoped for if the cells
contained hemoglobin composed of two domain, two
heme chains and a tetramer with two kinds of chains.

Double-stranded cDNA was prepared, inserted into
M13mp8 and cloned in JM103. A Northern blot with
nick-translated total cDNA showed one major band at
1200 bp and a weak band at 700 bp. One clone (AR12),
with an insert of about 300 bp, hybridized strongly
with poly(A)+ RNA of about 1200 bp. This insert
was cleaved by ClaI to give two fragments, which were
cloned and sequenced completely by the Sanger
dideoxy procedure. The sequences obtained provide
the amino acid sequence of the first 129 residues of
one domain, which shows a striking correspondence to
the known sequences (Como and Thompson, 1980;
Furuta and Kajita, 1983) of dimeric and tetrameric
globin chains from the related clam Anadara trapezia.
Both the proximal and distal histidines are present and
71% of the residues between them are identical to those in the corresponding positions in one of the two
clam globins. In contrast, only 36% of the corre-
spanding residues in the human a and b chains are identical. We have now prepared a new cDNA library
in XgtI0 from the poly(A)+ RNA and have isolated a
900 bp insert that hybridizes strongly with AR12. This clone should make possible the determination of the
complete primary structure of the two-domain hemo-
globin and provide a suitable probe for screening of a
genomic library.

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PUBLICATIONS
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Summary: In the past year, our work on the human
mitochondrial genetic system has developed in two
main directions: the identification of the polypeptides
encoded in mitochondrial genes and their functional
assignment, and the dissection of the mechanisms and
enzymatic machinery involved in the processing of
mitochondrial RNA. Substantial progress has been
made in both directions. Thus, the mitochondrial
translation products corresponding to four additional "unidentified reading frames" (URFs) of human mitochondrial DNA have been identified by the use of antibodies directed against synthetic peptides predicted from the DNA sequence. Furthermore, the mitochondrially-synthesized polypeptide corresponding to apocytochrome b has been identified by the isolation of the human b-c1 complex and characterization of its subunit composition and site of synthesis.

In a further major development, the products of six unidentified genes of human mitochondrial DNA have been shown to be components of the rotenone-sensitive NADH-ubiquinone oxidoreductase by immunoprecipitation experiments using peptide-specific antibodies and antibodies against the highly purified beef heart enzyme complex and by enzyme fractionation studies. This finding has solved what has been a total mystery since the discovery of the URFs, which make up more than half of the informational content of mammalian mitochondrial DNA. This discovery will have important implications, both in the context of the process of mitochondriogenesis and for the studies on the origin and evolution of mitochondrial DNA. Other immunoprecipitation experiments using peptide-specific antibodies have provided direct evidence that the product of another URF, URFA6L, is a component of the mitochondrial H+-ATPase. This finding has given support to the idea that the URFA6L product is equivalent to subunit 8 of the yeast H+-ATPase, as suggested by a small but significant amino acid sequence homology and other structural similarities. After these advances, a single URF of mammalian mitochondrial DNA still remains unidentified in its function.

In another area of mitochondrial research, several aspects of the mechanism of mitochondrial RNA synthesis and processing in HeLa cells have been investigated both in isolated organelles and in vivo. Thus, an event of premature termination of transcription of the 16S rRNA gene, which is promoted by actinomycin D and occurs both in vivo and in isolated organelles, has been analyzed in detail. This premature termination results in the formation of a discrete RNA species, RNA 16a, corresponding to the 5'-proximal 40% of the 16S rRNA gene, which is, interestingly enough, polyadenylated. An important observation relevant to the control of rRNA and mRNA synthesis in mitochondria is that actinomycin D can cause an almost complete block of 16S rRNA synthesis, mostly due to premature termination, still allowing a substantial transcription of the heavy strand genes situated downstream from the rRNA genes. These results strongly suggest that the synthesis of the rRNAs and mRNAs in HeLa cell mitochondria are independently controlled at a level distinct from that of termination of the transcripts at the 3' end of the 16S rRNA gene. This evidence, therefore, provides support for the existence of a control at the level of initiation, as implied in the model of two overlapping transcription units with distinct promoters, previously proposed by this laboratory. Further support for this model comes from a series of observations made on isolated organelles, which indicate that synthesis of rRNA and mRNA can be independently modulated under a variety of experimental conditions.

For other investigations, it has been shown that the processing of the COI/mRNA precursor (RNA 6) to mature mRNA (RNA 9) occurs in a single step by excision of the leader portion, which accumulates in vivo and in isolated organelles and is, in part, polyadenylated. This RNA species (which we have designated RNA 9L) is complementary to the four clustered tRNAs encoded in the L strand and to the template sequence corresponding to the origin of L-strand synthesis. Therefore, it has a highly structured configuration, which causes an unusual behavior in polyacrylamide gels.

18. Characterization of a Discrete Polyadenylated RNA Species Resulting from Premature Termination of Transcription of the 16S rRNA Gene Promoted by Actinomycin D

Julio Montoya, George L. Gaines, Michael P. King, Giuseppe Attardi

The electrophoretic pattern of mitochondrial RNA from HeLa cells labeled either in vivo or in isolated organelles in the presence of actinomycin D shows a polyadenylated RNA species (component 16a) migrating slightly faster than RNA 16. The mitochondrial DNA sequence coding for this RNA has been localized by mapping and sequencing studies to the rDNA region. The 5' end of this RNA 16a is immediately adjacent to the 3' end of the tRNA Val gene, and the RNA extends for approximately 645 nt downstream; therefore, this sequence corresponds to the proximal 40% of the 16S rRNA gene.
$S_1$ mapping experiments were carried out between RNA labeled in isolated organelles in the absence of actinomycin D and eluted from sequential slices of the region of the gel surrounding the position of RNA 16a, and appropriate restriction fragments. The results clearly indicated the presence of nascent chains having a common 5’ end, corresponding to the 5’ end of 16S rRNA, and arrested at various positions along the gene. When the isolated organelles were labeled in the presence of actinomycin D, the nascent chains were extended up to a position corresponding to the 3’ end of RNA 16a, resulting in the formation of this discrete species.

A quantitation of the effect of actinomycin D on the relative amounts of different RNA species labeled in vitro showed that with increasing concentrations of the drug, there was a progressive decline in the labeling of 16S rRNA, so that only 3% of residual labeling of this RNA was observed after treatment with 1.4 µg/ml of actinomycin D. By contrast, there was a substantial increase in the labeling of RNA 16a in the presence of the drug. The effect of actinomycin D on the labeling of RNA 16, taken as representative of the mRNAs, was much less pronounced relative to that of 16S rRNA; thus, RNA 16 was still labeled to the extent of more than 40% of the control in the presence of 1.4 µg/ml of the drug.

These results strongly suggest that synthesis of the rRNAs and mRNAs in HeLa cell mitochondria are independently controlled also at a level distinct from that of termination of the transcripts at the 3’ end of the 16S rRNA gene; in fact, the evidence supports the existence of control at the level of initiation, as implied in the model of two overlapping transcription units with distinct promoters.

19. **Analysis of the Leader of the mRNA for Cytochrome Oxidase I (9L)**

*George L. Gaines*

Two-dimensional gel electrophoresis of RNA labeled in isolated mitochondria (Gaines and Attardi, 1984a, 1984b) or in vivo has revealed the presence of an interesting species migrating distinctly from the normal diagonal. RNase H digestion of this RNA (called 9L) has showed that it is very probably a linear RNA. Furthermore, fine mapping using both $S_1$ nuclease techniques and primer extension has showed that 9L corresponds to the antisense of a cluster of tRNAs surrounding the light strand origin of replication, directly 5’ to the known mRNA for the gene COI. The existence of this RNA in vivo provides insights into the way mitochondria process their transcripts, possibly pointing to a connection between transcription and the control of DNA replication in these organelles.

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20. **Investigations on the Control of Processing of Mitochondrial RNA**

*George L. Gaines*

Using the "in organelle" technique of labeling, we have been able to specifically label the 3’ poly(A) or oligo(A) tracts on the mitochondrial RNA species. Actinomycin D at moderate concentrations blocks transcription in isolated mitochondria without stopping the addition of [$\alpha$-32P]ATP to the 3' ends of pre-existing mRNAs and rRNAs. We are currently studying the cleavage of the primary transcript and its relationship to the process of polyadenylation.

21. **Identification of the Products of Unidentified Reading Frames 2, 4, 4L and 5**

*Paolo Mariuttini, Anne Chomyn*

Using the same procedure that was used to identify the products of unidentified reading frames (URFs) 1, 3 and A6L, and of the ATPase 6 gene (Chomyn et al., 1983; Mariottini et al., 1983), we have identified the translation products of URFs 2, 4, 4L and 5. Peptides corresponding to portions of the URFs predicted from the mtDNA sequence were coupled to carrier protein and used to immunize rabbits. (Peptides and antisera were prepared in the laboratory of Dr. R. F. Doolittle at the University of California at San Diego.) The antisera were used to precipitate from an SDS lysate of mitochondria the specific translation products corresponding to the individual reading frames. Thus, anti-URF2 antibodies precipitated component 11, anti-URF4 antibodies precipitated components 4 and 5, and anti-URF4L antibodies precipitated component 26.

The antisera made against an URF5 carboxy-terminal peptide were unsuccessful in precipitating a
specific component from a mitochondrial lysate; those made against an amino-terminal peptide were able to precipitate weakly component 1. To gain additional evidence that component 1 is the product of URF5, we analyzed a trypsin digest of this component. The predicted amino acid sequence of the URF5 product indicates that, on digestion with trypsin, a dipeptide, methionyl-lysine, would be produced. Only the products of two other reading frames of human mtDNA—URF6L and URF2, which have already been identified—are predicted to yield a methionyl-lysine tryptic fragment upon tryptic digestion. It was found that one of the tryptic fragments of component 1 did indeed comigrate with methionyl-lysine in two-dimensional fingerprinting, thus confirming the identification of the component 1 as the URF5 product.

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22. Identification of Human Apocytochrome b

Claus-Jens W. Doersen

Cytochrome b is an integral component of complex III, the coenzyme QH2-cytochrome c reductase segment of the mitochondrial respiratory chain. In yeast and Neurospora, the apoprotein of cytochrome b has been demonstrated to be mitochondrially synthesized and encoded in mitochondrial DNA (see review by Borst and Grivell, 1978). The homologous gene has been found in the human mitochondrial DNA sequence (Anderson et al., 1981), but the corresponding mitochondrially-synthesized polypeptide has, until now, not been identified in HeLa cells.

The cytochrome b-c1 complex (complex III minus the non-heme iron protein) was isolated from human heart mitochondria essentially according to the procedure described by von Jagow et al. (1977). SDS/8 M urea polyacrylamide slab gel electrophoresis revealed that the components of the human cytochrome b-c1 complex migrated with similar electrophoretic mobilities to those of bovine complex III prepared according to the traditional procedure of Rieske (1967), with the expected absence of the non-heme iron protein in the cytochrome b-c1 complex pattern. In order to determine which mitochondrially-synthesized polypeptide is present in the cytochrome b-c1 complex, human heart mitochondria were mixed with mitochondria isolated from HeLa cells pulse-labeled with 35S-methionine in the presence of the cytoplasmic protein synthesis inhibitor cycloheximide and chased with cold methionine in the absence of cycloheximide. The cytochrome b-c1 complex was isolated from this mitochondrial mixture and the polypeptide composition was analyzed by polyacrylamide slab gel electrophoresis, followed by visualization of the components by silver staining or fluorography to detect the 35S-labeled components. Fluorography of SDS/8 M urea gels revealed a single labeled band corresponding to HeLa cell mitochondrial components 7, 8, 9, and 10, using the nomenclature of Ching and Attardi (1982). This band could be further resolved in using a 15–25% polyacrylamide gradient/SDS gel; using this system, the predominant 35S-labeled species in the isolated cytochrome b-c1 complex migrated with the electrophoretic mobility of component 8. (The significance of two minor bands with mobilities similar to those of components 7 and 9 is unclear and will require further investigation.) On the basis of the co-migration of the 35S-labeled polypeptide with a polypeptide (visualized by silver staining) with the expected mobility of apocytochrome b and its mitochondrial site of synthesis, apocytochrome b has been assigned to component 8 of the mitochondrially-synthesized polypeptides of HeLa cells.

References:
23. Immunoprecipitation of the Whole Cytochrome c Oxidase Complex by Antipeptide Antibodies

Paolo Mariottini, Anne Chomyn

We have previously determined (Biology 1984, Abstract No. 13) that antibodies directed against the synthetic C-terminal undecapeptide of cytochrome c oxidase subunit II (COII) precipitate from a Triton X-100 lysate of mitochondria the seven subunits which had been described in human cytochrome c oxidase (Hare et al., 1980). Furthermore, using different protocols of pulse labeling and pulse chase, we have shown that, of the three mitochondrially-synthesized subunits of cytochrome c oxidase, subunit I is the slowest to become incorporated into the enzyme complex. By the end of an 18-hour chase after a two-hour pulse labeling, all the labeled subunit I, subunit II and subunit III have become part of the complex.

Since a larger number of subunits than seven have been identified recently in mammalian cytochrome c oxidase, the above experiments have been extended. To visualize all the subunits of cytochrome c oxidase, both intra- and extramitochondrially synthesized, the cells were labeled in the absence of any inhibitor for approximately 22 hours with 35S-methionine. Under improved conditions of electrophoresis, it was shown that the COII C-terminal-specific antibodies precipitated all of the 13 subunits that have been described to constitute the mammalian cytochrome c oxidase (Kadenbach et al., 1983). Furthermore, it was observed that antibodies specific for an amino-terminal decapeptide, while able to precipitate free COII, were unable to precipitate COII assembled in a complex. These results strongly suggest that, in the native complex, the COOH-terminal peptide of COII is exposed to antibodies, whereas the NH2-terminal peptide is not accessible. These findings are, therefore, consistent with other evidence indicating that a portion of subunit II is buried in the complex within the inner mitochondrial membrane, while another portion protrudes out of the membrane, and with the existence of a concentration of hydrophobic amino acids in the amino-terminal portion of the predicted amino acid sequence.

References:

24. Functional Assignment of the Products of the Unidentified Reading Frames of Human Mitochondrial DNA

Anne Chomyn, Paolo Mariottini, M. W. J. Cleeter1, C. I. Ragan1, R. F. Doolittle2, A. Matsuno-Yagi3, Y. Hatefi3, Giuseppe Attardi

With the aim of determining whether any of the URF products are components of a multimeric protein complex, immunoprecipitation experiments using URF-specific antibodies were carried out. For this purpose, the same conditions that have allowed the precipitation of the 13 subunits of the intact cytochrome c oxidase from a Triton X-100 lysate of HeLa cell mitochondria by antibodies against a carboxy-terminal peptide of cytochrome c oxidase subunit II were used. Under these conditions, in addition to the production of URF4L, anti-URF4L antibodies precipitated specifically five other URF products—those of URF1, URF2, URF3, URF4 and URF5. This result suggested that all six URF products are components of a protein complex. In an effort to identify this complex, antisera directed against highly purified rotenone-sensitive NADH-ubiquinone oxidoreductase (Complex I) from beef heart were tested on the HeLa cell mitochondrial translation products. These antisera succeeded in immunoprecipitating very effectively and specifically the six URF products mentioned above from a Triton X-100 or deoxycholate mitochondrial lysate. The same six URF products were greatly enriched in a human submitochondrial fraction enriched in NADH-Q1 and NADH-K3Fe(CN)6 oxidoreductase activities. Therefore, it can be concluded that the six URFs encode components of the rotenone-sensitive NADH-ubiquinone oxidoreductase. It is proposed that the designations of URF1, URF2, URF3, URF4, URF4L, URF5 and URF4L be changed to ND (for NADH dehydrogenase), ND2, ND3, ND4, ND4L, ND5 and ATPase 8, respectively.

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25. Cloning of Human Nuclear Genes that Encode Mitochondrial Proteins

Jane Houldsworth

A number of proteins that are coded for by nuclear genes and synthesized on cytoplasmic ribosomes are essential for the assembly and function of mito-
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chondria. This project is involved with identifying the human nuclear gene(s) that encodes the ADP-ATP carrier of the inner mitochondrial membrane. This protein transports ATP out of the matrix space into the intermembrane space of mitochondria, and ADP in the reverse direction, so that it is indirectly involved in the oxidative phosphorylation process occurring in mitochondria. Identification of the nuclear gene(s) that encodes this protein will aid in the understanding of nuclear control over mitochondrial energy metabolism and, in general, of gene expression in human cells.

A cDNA library in pUC9 has been prepared from HeLa cell polysomal poly(A)+ RNA. Recombinants containing inserts specifying the ADP-ATP carrier protein will be identified in two ways. First, the library will be screened by colony hybridization with a cDNA probe for the homologous gene product in Neurospora crassa. RNA transfer hybridizations of human poly(A)+ RNA with this probe under relatively stringent conditions have revealed the presence of hybridizing mRNA species. Second, the ability of recombinant clones (identified using the above procedure) to hybridize to mRNA that directs in vitro translation of the ADP-ATP carrier will be tested. In this case, antisera to bovine ADP-ATP carrier will be utilized to immunoprecipitate the homologous in vitro synthesized human protein. A human genomic library will be screened with the identified cDNA species, and hence the organization of the nuclear gene(s) that encodes this protein crucial for mitochondrial function will be investigated.

26. Representation of the Multiple mRNA Species for Dihydrofolate Reductase in Different Mouse Tissues

Jiyoung K. Yang

One of the most intriguing observations made concerning the expression of the mammalian dihydrofolate reductase (DHFR) gene is the occurrence of multiple species of mRNA coding for this protein. These mRNA species all contain the coding sequence near the 5' end and differ in the length of the 3' untranslated region. In man, three major mRNA species with molecular sizes of 3.8 kb, 1.0 kb and 0.8 kb have been described (Morandi et al., 1982); in mouse, the four major species have sizes of 1.6 kb, 1.2 kb, 1.0 kb and 0.75 kb (Setzer et al., 1980).

With the aim of investigating the possible tissue specificity of the different DHFR mRNAs, poly(A)-containing RNA from various mouse tissues was analyzed for its content in the different mRNA species. The human DHFR cDNA plasmid pH84 (Morandi et al., 1982), 32P-labeled by nick translation, was used to probe the RNA samples transferred by electroblotting onto positively-charged nylon membranes (BioRad). A great variation of over a tenfold range was observed in the overall DHFR mRNA concentration in the poly(A)-containing RNA of different tissues. Furthermore, substantial differences were observed in the relative proportions of the various mRNA species; in particular, the ratio of the two most abundant species, the 1.6 kb and the 0.75 kb, varied between 0.6 and 5 in different tissues.

References:

27. Involvement of Small Polydisperse Circular DNA in Gene Amplification in Methotrexate-Resistant Human Cells

Barry J. Maurer

Small polydisperse circular (SPC) DNA has been found in numerous eukaryotic cells. These small covalently closed circular DNA molecules range in size from less than 1 kb to greater than 100 kb, share homologies with both repetitive and nonrepetitive chromosomal sequences, and may arise as the result of replicon "misfiring" (Kunisada and Yamagishi, 1984). The specific origin and possible role of SPC DNA in DHFR gene amplification in human cells exposed to methotrexate are being investigated. Quantitation of DHFR-related SPC DNA from unamplified HeLa S3 and DHFR gene-amplified 6A3 cells will indicate whether SPC DNA is produced from all genomic sequences or from a more limited subset. The effects of the addition or removal of methotrexate on the number of DHFR-related SPC molecules will also provide insight into the role they may play in the initial steps leading to DHFR gene amplification and in the maintenance of existing amplified DNA copies.

References:
28. Transcription Rates of Minor Upstream DHFR Initiation Sites

Barry J. Maurer

Recently, the main transcription initiation site for the human DHFR gene has been mapped to position -71 relative to the first nucleotide of the DHFR reading frame. Approximately 99% of all DHFR-specific polysomal polyadenylic acid-containing RNAs initiate at this site. However, ~1% of polysomal polyadenylic transcripts and ~11% of nuclear polyadenyl transcripts initiate at six or more additional sites between positions -449 to -480 upstream of the DHFR gene (Masters and Attardi, 1985). While low abundance upstream-initiating transcripts may play in DHFR regulation, experiments are being conducted to determine the transcription rates from the upstream initiation sites and the main initiation site using DHFR gene-amplified 6A3 cells.

To understand the role that upstream-initiating transcripts may play in DHFR regulation, experiments are being conducted to determine the transcription rates from the upstream initiation sites and the main initiation site using DHFR gene-amplified 6A3 cells.

References:

PUBLICATIONS


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Summary: In our laboratory we take a combined genetic and biochemical approach to the study of DNA replication. We work primarily with yeast because it is a good model for higher eukaryotic cells and because of the versatility of the experimental system. Our overall goal is to understand how initiation of DNA synthesis is controlled in the eukaryotic S phase and what proteins are involved in DNA replication. In order to understand these processes, it is essential to know what gene products are involved and how they are regulated.

In the past we have used in vitro replication systems to identify the proteins and DNA sequences involved in replication (Celniker and Campbell, 1982).
Recently, we have purified several proteins by biochemical assay that are thought to be involved in replication. We have made antibody and cloned the corresponding genes by screening an expression vector library using the antibodies as probes. The first gene isolated was for DNA polymerase I. Gene disruption experiments indicate it is essential for replication. This is the first eukaryotic polymerase ever cloned. DNA sequencing should prove invaluable for structure/function studies. The second gene isolated is for a single-stranded DNA binding protein that may interact with polymerase during replication. Mutants in this gene have not yet been constructed. This reverse genetics approach will be extended to the remaining primase, RNase H and helicase activities important for replication in the near future.

As to the initiation problem, we have further refined our functional map of sequences likely to be chromosomal origins of replication, namely autonomously replicating sequences (ARS). Furthermore, we have identified a protein that binds to the 11 base pair consensus sequence within all yeast ARS. Application of reverse genetics to this protein should tell us if these sequences participate in replication or transcription, another role sometimes ascribed to them. A by-product of these studies was the development of a flow cytometric assay for yeast cells, that is, the ability to measure yeast cells in the cell sorter. This has been used to assess plasmid stability and cell cycle regulation of expression of the single-stranded binding proteins.

Another project has focused on the prokaryotic model of replication. The dnaA protein of E. coli is analogous to the yeast ARS-specific protein in that it binds to the E. coli origin of replication and facilitates initiation there. We have studied the initiation of plasmid replication in bacteria and are now investigating the role of the dnaA protein in that system (Conrad and Campbell, 1979). This offers a paradigm for the yeast work and is an interesting regulatory system in which a small RNA molecule serves as a negative regulator of initiation. Amplification within the chromosome is also being studied.

Finally, we have instituted a program of in vitro mutagenesis of an unrelated protein, yeast iso-1-cytochrome c, in collaboration with some of our colleagues in the Division of Chemistry.

References:

29. Cloning of the Yeast DNA Polymerase I Gene
Lianna Johnson, Martin Budd, Judith L. Campbell

The polymerase I gene of yeast has been isolated by screening a λgt11 library with antibody against polymerase I. The gene has been characterized by overproduction in yeast using a cloned YEp24 vector, and overproduction in E. coli using a T7 promoter. The gene has been disrupted by inserting the URA3 gene into an internal fragment of the polymerase gene, transforming a ura3/ura3 diploid, then sporulating and dissecting the transformants. 2:2 viable:inviable segregation was observed with the viable spores being ura+. DAPI staining of the dead spores shows the terminal phenotype to be similar to cdc8 strains, cells in which the nucleus had migrated through the isthmus between the mother and daughter cell (Johnston et al., 1984).

Further experiments include functional characterization and sequencing of the polymerase gene. Purification of polymerase has yielded a polypeptide of molecular weight 150 kilodaltons, which corresponds to a 5 kb DNA fragment. Since the gene is on a 9 kb fragment, deletion studies are being done using nuclease Bal31. To characterize the deletions, the Bal31-treated DNA is transformed into a diploid strain with the polymerase gene disrupted with the LEU2 gene. The deletions contain a functional polymerase gene if 4:0 viable:inviable segregation is obtained after sporulation and dissection of the transformed diploid. Deletions created in this manner will be used for sequencing and for determining the minimal functional sequence.

Experiments to create a temperature-sensitive and an aphidicolin-resistant yeast polymerase I are also being done. The isolation will be done by using a partial diploid, a haploid with the polymerase gene disrupted with LEU2 and polymerase complemented on a URA3*YEp24 clone. The gene will be cloned into a CEN, TRPI vector followed by in vitro mutagenesis. After transformation with the mutagenized plasmid, the resulting transformants will be tested for temperature sensitivity to eliminate mutants unlinked
to polymerase. They will be screened for ura-, and screened again for temperature sensitivity to detect polymerase mutants.


30. Studies of a Gene Involved in DNA Replication

Martin Budd

A set of 12 mutants defective in DNA replication in vitro has been isolated (Kuo et al., 1983). One mutant, dna154, is defective in replication both in vitro and in vivo at 36°C. When dna154 cells are incubated at 36°C for 3 hr, they exhibit a terminal phenotype analogous to cdc2 strains, budded cells in which the nucleus has migrated to the isthmus but has not entered the daughter cell. The DNA154 gene has been cloned by complementation from a YCp50 library and is contained in a 4.5 kb fragment, as has been shown by deletion studies. The clone was shown to code for the Dnal54 protein by use of the marker rescue technique in which a fragment containing only the DNA154 gene and the β-galactosidase gene has been constructed which gives functional α-galactosidase activity in E. coli. However, in yeast the fusion protein does not have functional α-galactosidase activity, but does complement dna154 strains. The gene fusion offers a way to purify large quantities of a protein which has the same activity as the Dnal54 protein. The DNA154 clone and gene fusion can be used to determine whether the protein is involved in DNA precursor synthesis, DNA replication, or possibly nuclear transport.


31. The CDC8 Gene of Yeast Encodes Thymidylate Kinase

Ambrose Y.-S. Jong

cdc8 mutants of yeast have defects in cell division and in DNA replication. Temperature-sensitive cdc8 mutants shut off DNA synthesis immediately when shifted to the restrictive temperature. DNA molecules isolated from cdc8 cells at 36°C contain replication bubbles less than 3 µm reflecting a reduced rate of chain elongation. The cdc8 mutants have been studied intensively more than 12 years, but until recently little information has been accumulated about the gene product of CDC8. We have shown, namely, that thymidylate kinase is the product of the CDC8 gene of yeast as follows.

First, cdc8 mutants contain no detectable thymidylate kinase activity at either 23°C or 37°C. Second, the normal or even elevated thymidylate kinase levels can be restored to cdc8 strains when they are transformed with the wild-type gene, which we have cloned (Kuo and Campbell, 1983), and the fact that the level of enzyme is proportional to plasmid copy number and stability in transformants carrying the CDC8 gene cloned on a high copy number plasmid, provides strong evidence that the CDC8 product is thymidylate kinase. Finally, we have purified the enzyme approximately 5000 fold from a CDC8 plasmid-bearing strain that overproduces the activity 6 fold. The molecular weight, amino acid composition, and N-terminal amino acid sequence of thymidylate kinase are identical to those deduced from the DNA sequence of the cloned CDC8 gene (Jong et al., 1984).

In order to gain an understanding of the role of the thymidylate kinase in DNA replication, the enzyme has been further characterized (Jong and Campbell, 1986). Since cdc8 mutants are defective in replication in vitro where dNTPs are provided, the thymidylate kinase may play a second role, in addition to its catalytic function, as a structural part of the replication complex. Thus, the CDC8 protein may not only catalyze the synthesis of DNA precursors, but may also interact with the replication fork and is required for proper propagation of the replisome. Since we found that, in extracts, the enzyme is present in the nuclear/cytoplasmic fraction but absent from the mitochondrial fraction, it provides a good probe of a functional nuclear replisome.


32. Multiple Species of Single-stranded DNA Binding Proteins in Saccharomyces cerevisiae

Ambrose Y.-S. Jong

DNA replication is a very complicated process, requiring many proteins in addition to DNA polymerase
The role of single-stranded DNA binding protein (SSB) in DNA replication has been well established in prokaryotic systems. In eukaryotes, functions in transcription and RNA processing are also suspected. Our studies are aimed at systematically identifying all the single-stranded nucleic acid binding proteins of yeast with the goal of elucidating their individual functions through genetic, immunological and biochemical characterizations.

We have done a systematic search for yeast SSBs following the traditional affinity purification schemes (Jong and Campbell, 1985), and have identified major species of 50,000, 45,000, 31,000, 22,500, and 20,000 daltons and some smaller proteins. We have purified three of them to homogeneity—SSB-m, SSB-1, and SSB-2, with molecular weights of 20,000, 45,000 and 50,000 daltons, respectively. We have produced highly specific and high titer antibodies to SSB-1 and to SSB-2 and shown that not only do these two proteins not cross-react with each other, they also do not cross with SSB-m. We provide two lines of evidence to suggest that SSB-1 is involved in nuclear DNA metabolism. First, it stimulates yeast DNA polymerase I, the replicative polymerase. Second, the affinity-purified SSB-1 antibodies inhibit synthesis in our in vitro replication system.

SSB-2 is a new class of eukaryotic SSB on the basis of molecular weight, inhibition of DNA polymerase and antigenicity. We have not found any enzyme activity associated with this protein, such as DNA-dependent ATPase, topoisomerase or nuclease, but we observe that SSB-2 protein inhibits yeast DNA polymerase I when added in concentrations where SSB-1 stimulated optimally. SSB-m is found only in mitochondria and is the first mitochondrial SSB described. We have not yet purified the 31,000 or 22,500 dalton protein to homogeneity. Study of these proteins should be carried out in future investigations. Our goal is to use these proteins as reagents to clone the genes for the respective proteins, to mutagenize the genes in vitro, and to reintroduce the altered gene into yeast and thus assess the relationship of the role of these genes and of these proteins in vivo.

References:

33. Isolation and Characterization of Yeast SSB-1 Gene

Ambrose Y.-S. Jong

In order to define the individual roles of the multiple species of single-stranded DNA binding proteins in yeast, the proteins are being used to provide probes for isolating the corresponding genes and mutants.

A yeast genomic library constructed in phage λgt11 was screened using antibodies to yeast SSB-1, the SSB that stimulates DNA polymerase I and has been proposed to function in replication. Of 5 x 10^5 plaques screened, we identified 12 positive clones which should contain the SSB-1 gene fragment fused in lacZ region of λgt11. In order to identify the intact yeast gene, the insert fragment from one of the positive clones was isolated and used to screen a second yeast genomic library, in a high copy number plasmid YEp24. Strains carrying high copy plasmids containing SSB-1 gene overproduced SSB-1 mRNA by 10 fold and SSB-1 protein by threefold, as determined by RNA and protein blotting. Expression in E. coli was also demonstrated. Disruption of the gene is not lethal to yeast. Thus, either the function is dispensable or there is a compensatory activity (Jong and Campbell, manuscript in preparation).

34. Are Autonomously Replicating Sequences Origins of DNA Replication?

Kevin Sweder, Judith L. Campbell

While it is generally believed that yeast DNA replication occurs bidirectionally from multiple, specific origins of replication, no direct evidence of a specific DNA sequence requirement exists. However, several lines of circumstantial evidence tend to support the idea of specific origins of replication. Sequences that enable high frequency transformation and extra-chromosomal replication of any colinear DNA in yeast are called autonomously replicating sequences (ARS) and are good candidates for chromosomal replication origins (Stinchcomb et al., 1979; Struhl et al., 1979). In addition to the above properties, microscopy and fiber autoradiography indicate origin-to-origin distances of 10 µm to 60 µm, corresponding to approximately 400 origins per haploid genome and this is in good agreement with the estimate of the number.
determined by cloning of chromosomal sequences that mediate high frequency transformation.

The functional domains of ARS1 have been dissected using Bal31 and S1 mapping (Celniker et al., 1984; Kearsey, 1984) and shown to consist of three domains, which we have designated A, B, and C. Domain A consists of an 11 base pair (bp) consensus sequence present in ARS activity on centromeric plasmids (Srienc et al., 1985). Domain B immediately flanks domain A and is between 46-109 bp. Deletion of domain B drastically reduces the stability of the plasmids. Domain C, about 200 bp in size, flanks domain A on the opposite side from domain B. Domains B and C show little homology between different ARS. Our model for the function of the domains within ARS1 is that domain A is a protein recognition site crucial for DNA replication, analogous to the 9 bp dnaA protein-binding site of E. coli, and the sites recognized by the O protein of lambda phage, the T antigen of SV40, or the nuclear factor I of adenovirus. Domain B may contain the sequences required for proper temporal initiation during S phase and/or as attachment sites to ensure proper segregation. Deletions in domain C have been shown to affect stability of centromeric plasmids but its function remains unknown.

Our laboratory is currently involved in isolating proteins that interact directly with the ARS region. Since the function of such proteins is not known a priori, we have chosen to isolate them by their ability to bind the ARS and protect it from DNase I digestion (Galas and Schmitz, 1978). By using DNase I footprinting, we avoid assays that require either a highly purified protein or large amounts of the protein. Once we have completed purifying the protein that binds to the consensus sequence, we can biochemically characterize it. By producing antibodies to the protein, we can isolate the gene from a yeast library such as YCP50. Isolation of the gene will enable us to mutagenize the gene and by gene replacement to determine the role of the protein in the yeast cell cycle.

References:


Friedrich Srienc, James E. Bailey1, Judith L. Campbell

Flow cytometry measures certain cell features (fluorescence, light scattering) on a single cell level at high speed (1000-5000 cells/sec). A statistically reliable density function characterizing a cell population according to the measured parameter can be obtained in less than a minute. We have used flow cytometry for determination of plasmid stability in yeast. The method is based on detection of β-galactosidase-encoding plasmids in individual cells of Saccharomyces cerevisiae, enabling rapid and statistically accurate determination of the fraction of plasmid-containing cells in a cell culture (Srienc et al., 1983). This assay measures enzymatic cleavage of α-naphthol-β-D-galactopyranoside by intracellular β-galactosidase and trapping of the liberated naphthol by hexazoniumpararosaniline yielding a fluorescent, insoluble end product. For our studies, we used a set of plasmids encoding β-galactosidase under the control of the inducible GAL10 promoter.

We have used a set of deletion mutations in the ARS1 element of yeast to measure their effect on chromosome stability in yeast (Celniker et al., 1984). This work establishes the previously proposed existence of three domains in ARS1. Domain C, which we had previously inferred, but not proved, to be a part of ARS1 is now established. In addition, we show that increasingly large deletions of the domain have increasingly large effects, which was not realized before. Furthermore, we have provided the first positive evidence for the central importance of a 14 bp core sequence containing the ARS consensus element by showing that it has the ability to act as a replicator on a plasmid containing no other ARS1 flanking sequence (Srienc et al., 1985).

A growing cell population is typically heterogenous due to distribution of particular cells over different phases of the cell cycle. Flow cytometry measurements combined with cell sorting is a nonperturbative method of separation of cells according to cell cycle position. We have used this procedure to determine
cell cycle regulation of expression of a yeast single-stranded binding protein thought to be essential for replication.

References

36. Does dnaA Protein Participate in the DNA Replication of ColE1-type Plasmids?

Doreen Ma

The dnaA protein is an essential component in the initiation of replication from oriC (origin of E. coli chromosome) (Wechsler, 1978). This protein has been purified and used in an in vitro replication system to provide specific initiation at oriC (Fuller and Kornberg, 1983). Fuller et al. (1984) have proposed a consensus dnaA protein recognition sequence, 5'-TTATC(A)CAC(A)-3', which is repeated four times within the minimal oriC sequence. The consensus sequence is also present in a few other promoters and origin sites of different plasmids. A DNase I footprinting study by Fuller et al. (1984) has definitively demonstrated dnaA protein binding to the ori-region of pBR322. This result, together with mutational and biochemical studies, strongly suggests that dnaA protein participates in ColE1 replication (Abe, 1980; Polaczek and Ciesla, 1984).

Initiation of replication of ColE1-type plasmids is regulated by two trans-acting negative control elements. One of these elements, RNA I, when hybridized to RNA II, the preprimer, interferes with RNase H catalyzed processing of RNA II to become the mature primer. The other element is the repressor of primer (rop) gene product which is a small plasmid-encoded protein. With the results of our mutational studies, we have suggested that the rop gene product acts by enhancing the RNA I:RNA II interaction and disfavoring the RNA II:DNA hybrid formation (Moser et al., 1984). At present, we are developing an in vitro transcription system that faithfully mimics the in vivo process. This system synthesizes both the 108 nucleotide RNA I and the preprimer RNA II. Addition of RNase H cleaves the preprimer to a 555 nucleotide RNA that appears as a new band on a high resolution polyacrylamide sequencing gel. One model of dnaA protein participation involves the protein binding and stabilizing the DNA-RNA hybridization for primer processing. We would expect that addition of dnaA protein into the in vitro system would enhance the production of RNA II, thus intensifying the 555 nucleotide band. Other possibilities are being considered too.

In order to carry out the proposed in vitro studies, we need large amounts of the E. coli dnaA protein. Although a published procedure of purification exists (Fuller and Kornberg, 1983), the scheme is not reproducible and requires extreme care to achieve useful results. Therefore, we have worked out a more efficient purification. With dnaA protein purified, we will direct our attention to testing our proposal.

References:

37. Development of a Method for Improving the Stability of Amplified Foreign DNA in Bacteria

L. Elizabeth Bertani, Giuseppe Bertani1, Judith L. Campbell

The possibility of improving the stability of cloned foreign genes by transferring them first to the chromosome of E. coli and then amplifying them in situ is being investigated. We start with a hybrid plasmid consisting of the integration system of the temperate phage P2 cloned into the non-integrating plasmid pBR322. The hybrid is then introduced into a temperature-sensitive polA- host, so that extrachromosomal plasmid DNA can be eliminated by culturing at the nonpermissive temperature. Southern blot analysis of the resultant strains indicates that the hybrid integrates in single copy at the preferred attachment site of the phage.

Strains carrying a single integrated plasmid copy retain a low level of resistance to tetracycline. The integrated DNA can be amplified by selecting for in-
creasing resistance at the non-permissive temperature. Two independently-obtained strains examined so far have been found to be carrying 10 and 18 copies of repeated units of 41.4 and 46.5 kilobases, respectively. The repeated unit thus includes large amounts of surrounding DNA in addition to the integrated plasmid; as a result of amplification, the additional DNA corresponds to a 12-2496 increase in the size of the E. coli chromosome.

Although the single-copy integrants are very stable at all temperatures in the absence of antibiotics, the high copy number of the amplified derivatives cannot be maintained in the absence of selective pressure. The copy number of the larger repeat was reduced to four after 14 generations in medium lacking antibiotic. We hope to be able to stabilize the high copy number by introducing a RecA+ mutation into the host.

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38. The Use of "Nonperturbative," Site-directed Mutagenesis for the Study of Electron Transfer Mechanisms in Metalloproteins

Stephen Mayo, John H. Richards1, Harry B. Gray1, Judith L. Campbell

Recent advances in genetic engineering methodology have led to the design of proteins that provide new routes to the study of electron transfer mechanisms. Using site-directed mutagenesis (Zoller and Smith, 1983), we have optimized yeast iso-1 cytochrome c (Montgomery et al., 1978; Smith et al., 1979) for studying intramolecular electron transfer mechanisms via the ruthenium modification method (Margalit et al., 1985). Each mutation was designed to maintain the structural and functional integrity of the protein while providing for the efficient study of the effect of donor/acceptor distance, intervening medium, and donor/acceptor orientation on intramolecular electron transfer rates. Mutants were designed on an Evens and Sutherland PS300 (and interactive computer graphics system) using the protein engineering simulation capabilities of program BIOGRAF/I (written by S. L. Mayo, B. D. Olafson and W. A. Goddard III). Currently, we have completed the generation, isolation and characterization of three isostructural/isofunctional mutants of yeast iso-1 cytochrome c. The first two mutations, Cys102/Ser and Ser102/Thr, were designed to circumvent complications in the physical characterizations caused by the cysteine sulfhydryl. And the third mutation, His39/Lys (performed on the Thr102 mutant), was designed to provide convenient access to the other naturally occurring surface histidine (His33). We are now concerned with the design rationale and experimental manifestation of the above mutations and the biological and physical characterization of these systems. The work also addresses the potential of using protein engineering and computer graphics methodologies for the design of novel, metalloprotein-based, redox catalysts (for example, see Margalit et al., 1983).

References:

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PUBLICATIONS
Summary: We are interested in the organization and expression of the genome in higher animals. In recent years molecular cloning techniques have enabled us to carry out experiments at the level of individual genes.

We use sea urchin eggs, developing embryos and larvae as model systems in much of our work. The sea urchin has a small genome, which makes cloning comparatively less difficult. The animals are abundant on the Southern California coast, and gametes are easy to obtain in large numbers. The stages of early development have been described by classical and modern embryologists in considerable detail, both morphologically and biochemically. Several years ago we established at the Kerckhoff Marine Laboratory in Corona Del Mar a sea urchin maintenance system that supplies eggs and sperm for developmental experiments year round. In general, projects concerned with the molecular biology of sea urchin development are done in our Pasadena laboratory, while those at the Kerckhoff Marine Lab are centered on genome organization and evolution.

A basic feature of early sea urchin development is the presence in the egg of RNA molecules synthesized during oogenesis. Some of this RNA consists of maternal messenger RNAs, which are stored in the egg and utilized during early cleavage stages. An additional class of very long maternal RNA sequences, resembling nuclear RNA, was identified in this laboratory several years ago. We are continuing to characterize these sequences, which contain transcripts of repetitive as well as single copy genomic DNA.

We have developed methods of introducing cloned DNA into sea urchin eggs and embryos by microinjection with a continuously flowing needle—a sea urchin transformation system. This system is being exploited in a number of studies of gene regulation during embryogenesis. In particular, we are beginning to analyze the putative regulatory regions at the 5' ends of various sea urchin actin genes, which are expressed in a stage- and tissue-specific manner in young embryos. The transformation system is expected to be useful also in characterizing other recently cloned genes, which are active in embryogenesis.

In addition to projects of embryological interest, the lab continues analysis of sea urchin genes active in adult tissues, and of genome evolution. In all, our aim is to better understand the mechanisms of gene control in development and the relationships among DNA sequences in higher animals.

39. A Maternal Repetitive DNA Sequence Transcript in Sea Urchins

Frank J. Calzone, James J. Lee, Nga Le

All eggs contain stored maternal RNA for use in early embryo development. In the majority of poly(A)+ RNA molecules in the sea urchin egg, single copy DNA sequence transcripts are interspersed with repetitive DNA sequence transcripts and the RNA does not appear to be translatable. We have determined the structure of one interspersed maternal RNA, the 2109A-10 transcript, which contains a genomic repeat called 2109A. Nuclease S1 and exonuclease VII protection assays indicated that the 2109A-10 transcription unit generates a 3.7 kb primary transcript which does not appear to be spliced to produce a smaller RNA in the egg or embryo. This transcript does not contain any statistically significant large open reading frame. Therefore, we believe that the 2109A-10 transcript
cannot be a source of mRNA translated in the egg or early embryo.

Repetitive DNA sequence transcripts are localized in the 5' half of the 2109A-10 transcript. Two different repetitive elements were identified. One is moderately repetitive in the sea urchin genome and contains sequences homologous to the genomic 2109A repeat. The adjacent sequences are very highly repeated in the genome. An identical copy of one 180-nt sequence present in the high frequency repeat of the 2109A-10 transcript, approximately 500 nt from the start of transcription, appears to be shared by many other egg and embryo transcripts. Unlike other repetitive elements examined in the sea urchin, the 180-nt sequence is asymmetrically represented in RNA and thus may have functional significance. The prevalence of the maternal transcript is very low (375 copies/egg) and increases less than threefold after fertilization. Experiments in progress seek to determine the fate of maternal and embryonic 2109A-10 transcripts and to examine the expression of the transcript in embryo nuclei. Future work will also focus on a possible role for the 2109A-10 transcripts in the regulation of gene expression in the developing embryo.

40. Cloned Maternal RNA from a Single Individual

Laurie E. Klein

To facilitate anticipated future studies of interspersed maternal RNAs (see Abstract No. 39), I have made an oligo(dt)-primed cDNA library from poly(A)+ RNA isolated from the eggs of one individual sea urchin. The library, in the λgt10 vector, contains 130,000 recombinant clones. The average length of the cDNA inserts is 1.6 kb. A randomly primed cDNA library for the same poly(A)+ RNA is under construction.

41. The Fate of Embryonic Sea Urchin Cells during Development to the Adult Form

R. Andrew Cameron

We are studying the mechanisms by which specific patterns of gene activity are expressed in specific cell lineages during sea urchin development. While the general course of early embryo cell lineage formation is well known (Horstadius, 1939), the exact nature of cell origins and cell fates in later sea urchin embryos and larval stages is not. It is our goal to describe in detail the fate of early cleavage-stage blastomeres through larval development and metamorphosis into the juvenile sea urchin and to delineate specific larval regions that contribute to structures that persist in the juvenile. In addition to describing cell lineages in late development, these experiments will form a basis for later investigations of gene activity in late larvae and during metamorphosis.

We have perfected a technique for the injection of individual, 16-cell stage macromeres with fluorescent dyes while allowing normal development. A complete determination of the fate of vegetal pole blastomeres is under way, as these marked cells will be followed through metamorphosis. Using the extensive, routine cell manipulation techniques developed for sea urchin embryos (for example, surgical blastomere replacement methods), we intend also to substitute cells of the animal pole blastomeres and of the micromere group with dyed ones and follow their fates.

Reference:

42. Expression of a Cloned Heat Shock Fusion Gene in Sea Urchin Embryos

Andrew P. McMahon, Thomas J. Novak

In the sea urchin transformation system developed in this laboratory, cloned DNA molecules injected into eggs were found to persist and accumulate in embryos (Flytzanis et al., 1985; McMahon et al., 1985). In order to find out whether the exogenous DNA would be expressed in the developing embryos, we microinjected a fusion gene construct in which the coding sequence for bacterial chloramphenicol acetyltransferase (CAT) had been placed under the control of the regulatory region of the Drosophila gene for the 70 kilodalton heat shock protein (Di Nocera and Dawid, 1983). Pluteus-stage embryos developing from the injected eggs were analyzed for the presence of CAT enzyme, and were found to express the CAT gene. After the embryos were exposed to high temperature, the amount of CAT enzyme activity increased eight to tenfold. Extracts from control (uninjected) embryos had no CAT activity. The injected DNA was present in experimental embryos in high molecular weight concatenates, and during development was amplified about 100 fold. We calculated that amplified
sequences were responsible for all or most of the induced CAT enzyme activity.

References:

43. Analysis of Transformed Sea Urchin Embryos by Immunocytochemistry

Barbara R. Hough-Evans

It has been shown recently (McMahon et al., 1984, and Abstract No. 42) that cloned genes injected into the unfertilized sea urchin egg can be expressed in the developing embryo. It is not yet known whether these genes are expressed in a tissue-specific manner. We are exploring techniques of fixation and sectioning in order to examine embryos immunocytochemically. The cloned DNAs injected will include the gene for bacterial chloramphenicol acetyltransferase (CAT) attached to the sea urchin regulatory sequences to be tested. Our aim is to detect the CAT enzyme in embryo sections using a double antibody method, with the second antibody labeled with fluorescein or peroxidase. In this way it should be possible to determine the cellular or tissue location of introduced genes that are expressed during embryo development.

Reference:

44. Expression of an Actin Gene Transferred to Developing Sea Urchin Embryos

Constantin N. Flytzanis, Lisa S. Gobar

The actin gene family of the sea urchin Strongylocentrotus purpuratus provides us with an excellent model for the study of differential gene expression during the early stages of embryo development. As previously shown (Lee et al., 1984), there are six active S. purpuratus actin genes and two pseudogenes. The six genes are expressed at different developmental stages (Shott et al., 1984) and, as shown recently, in different cell lineages of the early embryo (Cox et al., 1985). Using the gene transfer system for sea urchins that we developed recently (Flytzanis et al., 1985; McMahon et al., 1985), we have focused our efforts on understanding the mechanisms underlying the ontogenic regulation of one member of the actin family, the CyIIla gene (Shott-Akhurst et al., 1984). The CyIIla gene is transcribed exclusively in embryonic and larval stages (Shott et al., 1984) and only in the cells that comprise the aboral ectoderm of the embryo. We constructed a plasmid that carries the 5' flanking sequences and part of the coding region of the CyIIla gene, fused to the structural gene sequences of the bacterial enzyme chloramphenicol acetyltransferase (CAT). This plasmid was microinjected in the linear form into unfertilized eggs of S. purpuratus, and after fertilization the embryos were collected at different developmental stages. The expression of the injected genes was followed by assaying for CAT enzyme activity in extracts from pooled embryos. The injected DNA molecules undergo amplification during embryonic development, which was measured by hybridizing radioactively-labeled probes to the DNA extracted from pools of injected embryos. The results from a series of such experiments indicate that the injected DNA sequences are amplified in the embryos to the same extent as shown earlier (Flytzanis et al., 1985; McMahon et al., 1985), and that the CAT enzyme is found at the same developmental stages as CyIIla actin transcripts. These results suggest that the cis-acting elements necessary for the ontogenic regulation of the CyIIla gene are present on the 5'-flanking sequences used to construct the CyIIla CAT. At present we are focusing our efforts in collaboration with Barbara Hough-Evans, on determining whether the expression of CAT in the injected embryos is cell lineage specific by using antibodies to the bacterial enzyme. We are also trying to find the necessary regulatory elements on the CyIIla CAT, by deleting sequences from the 5' end of the actin flanking region and injecting the deleted plasmids.

References:
45. Transformation of Sea Urchin Embryos by Nuclear Microinjection

Robert R. Franks

We have devised a method for the introduction of DNA by microinjection into nuclei of early zygotes of the sea urchin *Lytechinus variegatus*. Our purpose is to determine whether injection directly into the nucleus will give rise to a larger proportion of transformed larvae and juveniles than does injection into the cytoplasm. For these studies, the plasmid CyIIIA CAT (see Abstract No. 44) was used. This plasmid contains the promoter region of a *Strongylocentrotus purpuratus* gene encoding a developmentally-regulated cytoplasmic form of the actin protein (CyIIIA; Shott et al., 1984) fused to sequences encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT). Expression of the injected sequences was followed by measuring CAT enzyme activity in extracts of embryos at different stages of development. In all groups of embryos tested the exogenous DNA displayed accurate temporal regulation with respect to the actin promoter. The level of CAT activity was comparable in early embryos derived from cytoplasmically- and nuclear-injected zygotes.

We also tested for the presence of the foreign DNA in individual two-week-old larvae, just before metamorphosis occurs. Using dot blot assays, we found that the injected sequences persist in about 41% of the larvae descended from cytoplasmically-injected zygotes. A higher value, about 53% positives, was obtained for the nuclear group. The level of amplification of the injected DNA molecules also differed for the two groups of larvae. In more than 90% of cytoplasmic positives the DNA had been amplified 10 to 600 fold, while few showed greater than a 700-fold increase. In 30% of the nuclear positives the DNA had been amplified 700 to 3300 fold. We interpret this to mean that after nuclear injection the exogenous DNA is present in more larval cells.

During metamorphosis most of the larval tissues are destroyed, and the juvenile sea urchin arises from the imaginal rudiment. If the exogenous DNA is to be carried through to the adult, it must be present in the imaginal cells. We raised larvae derived from nuclear-injected and cytoplasmically-injected zygotes through metamorphosis, and reared juvenile sea urchins. DNA was extracted from individual juveniles about two months after metamorphosis. We tested genome blots of each individual for the presence of exogenous DNA sequences. Five to ten percent of juveniles in the cytoplasmic group were found to contain injected sequences, while 23-26% of those in the nuclear group were positive for the injected DNA. It appears, therefore, that a significantly greater number of larvae arising from nuclear-injected zygotes contain the DNA in most or all of their cells. The high occurrence of mosaicism among cytoplasmically-injected individuals would suggest that the injected DNA becomes associated with nuclei and integrated into genomic DNA at different times during development, and in only some of the embryonic cells. Based on our results, an effective means of increasing the number of cells containing introduced DNA sequences is to inject the DNA directly into the zygote nucleus.

Reference:

46. Structure and Regulation of the Cyl Actin Gene in the Sea Urchin

Karen S. Katula

The genome of the purple sea urchin *Strongylocentrotus purpuratus* contains eight nonallelic actin genes. It has been shown that at least six of these genes have unique temporal and spatial patterns of expression (see Abstract No. 44). The Cyl gene is a cytoskeletal actin that is expressed in all the cell types of the embryo except for the aboral ectoderm and in every adult tissue examined. Very little Cyl actin transcript is observed in the embryo until 14 hours of development. At this time there is a great increase in the number of Cyl transcripts, which continues until the end of embryogenesis.

The 5' cap site of the Cyl actin gene was determined using S1 analysis and primer extension. We found that the Cyl gene has an intron of approximately 1.3 kb in its 5' end. The 3' splice site is 25 bp from the Cyl gene ATG. The start of transcription was localized
to an "A" that is 53 bp from the 5' splice site. Both a TATA box and CAAT box have been found in the 5' flanking sequences.

To investigate the regulation of Cyl actin gene expression, we have constructed a fusion gene between 5' sequences of the Cyl gene and the bacterial enzyme, chloramphenicol acetyltransferase (CAT). Expression from this plasmid is tested for by injecting the plasmid into sea urchin eggs and assaying for CAT activity at various developmental stages. A plasmid containing 4 kb of sequence 5' to the actin ATG site fused to CAT was found to be expressed at 14, 24 and 48 hours but not at 10 hours of development. This is consistent with normal Cyl expression.

Plasmids are now being constructed that contain deletions in the 5' sequences to localize the DNA sequences required for developmental regulation of the gene.

47. Sea Urchin Actin Gene Linkage by Restriction Fragment Length Polymorphism Segregation

Joseph Minor, James J. Lee, Patrick S. Leahy

The linkage of the sea urchin actin genes is being studied by the observation of segregation of restriction fragment length polymorphisms (RFLPs, see Biology 1984, Abstract No. 30). The preliminary results are as follows. Actin genes Cyl and Cylla segregate their RFLPs evenly (approximately 1:1 ratio) and in all cases the RFLPs segregate as if the genes were linked. This confirms overlapping clones that put Cyl and Cylla in the same linkage group. In contrast, Cylla appears to be unlinked to Cyl-Cylla. Cylla exhibits some strange segregation behavior: one RFLP appears to be represented preferentially in the F1. Further work will increase the data-set on the above results and will also determine the linkage of the M gene.

48. Actin mRNA Accumulation in Sea Urchin Embryogenesis

James J. Lee, Frank J. Calzone

The number of molecules of mRNA representing each of five different actin genes was investigated in developing embryos of the sea urchin Strongylocentrotus purpuratus. Transcripts of the cytoskeletal actin genes Cyl, Cylla, Cyllb, and Cyllla, and of the muscle actin gene M, were measured in unfertilized egg and embryo RNAs of cleavage, blastula, gastrula, and pluteus stages. The measurements were obtained by probe excess titrations of these RNAs, using a set of single-stranded RNA probes each identifying the mRNA transcripts of a specific actin gene. As described by Lee et al. (1984) these mRNAs can be identified by their distinct 3' nontranslated trailer sequences. We confirmed earlier observations that the prevalence of actin mRNA in the unfertilized egg is low. Cytoskeletal actin genes Cyl and Cyllla each contribute 1-2 x 10^3 maternal mRNA molecules, and Cyllb contributes fewer than 2 x 10^2 mRNA molecules, while no detectable maternal mRNAs derive from cytoskeletal actin gene Cylla or the muscle actin gene M. We also found that at certain periods of development, transcripts derived from the individual cytoskeletal actin genes accumulate rapidly, with kinetics specific to each mRNA. Transcripts of the muscle actin gene were absent until after gastrulation, when the initial muscle progenitor cells are formed. At late stages of development each of the five genes studied was represented by 10^4-10^5 mRNA molecules per embryo. Together with the observations of Cox et al. (1985) (see Abstract No. 49), these measurements permitted us to calculate the levels of each actin mRNA species in the particular cell types in which each gene functions in the fully differentiated embryo.

References:

49. Cell Lineage-specific Expression of Actin Genes in Sea Urchin Embryos

Katherine H. Cox, Lynne M. Angerer, James J. Lee

We have determined spatial patterns of expression of individual actin genes in embryos of the sea urchin Strongylocentrotus purpuratus. Radioactively-labeled probes specific for each of five cytoplasmic-type (Cy) and the single muscle-type (M) mRNAs were hybridized in situ to sections of fixed embryos at several stages of development. M actin mRNA appears only late in development and is confined to a few cells associated with the coelomic rudiments, which later give rise to the adult sea urchin. The five Cy mRNAs fall into three sets whose time and sites of expression during development are highly distinctive. Different cell lineages express messages of one or more of these
sets, but never all three. Although all Cy actin mRNAs exhibit monophasic accumulation in the RNA of whole embryos during the course of development (Shott et al., 1984; Lee et al., 1985 and Abstract No. 48), such accumulation in many cases results from the summation of both increases and decreases in abundance within individual sets of cells. Within the genomic linkage group CyI-CyIIa-CyIIb, expression of Cy! and CyIIb appears to be coordinate and quite distinct from that of CyIIa. CyI and CyIIb are expressed in all lineages at some point in embryogenesis, but are confined mainly to oral ectoderm and portions of the gut of the pluteus larva. CyIIa mRNAs are restricted to mesenchyme lineages throughout late gastrula stage, and subsequently accumulate in parts of the gut. The CyIIIa and CyIIlb genes, which form a separate linkage group, are expressed only in aboral ectoderm and its precursors. Furthermore, CyIII messages are the only detectable actin mRNAs in this cell lineage after late blastula stage.

References:

50. Cloning of Sea Urchin Myosin Heavy Chain and Other Muscle-related Genes

Samuel J. Rose III, Marvin J. Rosenberg

We have begun a study of genes coding for proteins normally expressed in sea urchin muscle. Our goal is to compare the coordinate expression of these genes with each other and with the muscle-specific actin gene (Lee et al., 1984) already cloned.

A Drosophila genomic clone encoding the 3' two-thirds of the myosin heavy chain gene has been used to identify a lambda genomic clone from a sea urchin library under high criterion cross-hybridization conditions. An 800 base pair subclone of this lambda contains a coding sequence that is 75% homologous in nucleotide sequence and 70% homologous in amino acid sequence with the Drosophila myosin heavy chain gene. This subclone hybridizes to a 6.0 kb poly(A)+ mRNA from sea urchin muscle tissue and to only two bands on genomic Southern blots of DNA from different individuals. The gene appears to be single copy in sea urchins as it is in Drosophila but in no other animal yet analyzed. We have also optimized conditions for cross-hybridization of Drosophila alpha-tropomyosin and chicken M-creatine phosphokinase clones to sea urchin DNA. Both appear to be multigene families in the sea urchin.

A cDNA library from sea urchin lantern muscle RNA is now being prepared for subsequent screening using each of the above probes (and more as they become available). This should allow us to isolate muscle-specific gene subtypes from multigene families and to study transcript nucleotide sequences for evolutionary comparisons.

References:

51. Isolation and Characterization of cDNA Clones Encoding Hyalin

John J. Robinson

Hyalin is a major component of the extraembryonic protein coat, the hyaline layer, which surrounds the developing sea urchin embryo. The purified protein migrates as a single band of apparent molecular weight 330,000 on SDS-PAGE. Biochemical experiments have shown that hyalin synthesis is both stage and tissue specific, with synthesis occurring in ectoderm cells from the mid-blastula stage on (McClay and Fink, 1982). Using polyclonal antihyalin antibody we have isolated two distinct clones from a cDNA library constructed in the expression vector Agt11. In situ immunofluorescence and protein gel blot analysis clearly demonstrated that the antibody preparation reacts specifically with hyalin. Both clones encode fusion proteins that strongly react with antihyalin antibody but are immunologically non cross-reactive with each other. This latter result suggests that the cDNA inserts code for polypeptides that represent different parts of the hyalin molecule. An RNA gel blot reaction showed that the cDNA inserts hybridized to different transcripts, suggesting that hyalin is not a single, large polypeptide but rather is composed of at least two distinct subunits. We are currently studying the tissue specificity of hyalin gene expression.

Reference:
52. Characterization of the Major Spicule Matrix Protein Gene

Henry M. Sucov, Steve Benson¹, Fred Wilt¹

One of the best understood and most easily accessible lineages in the sea urchin is the micromere lineage. Formed as a quartet of particularly small cells at fourth cleavage at the vegetal pole of the embryo, these cells ultimately give rise to a protein-CaCO₃ skeletal structure known as a spicule. As primary cultures of isolated micromeres proceed to develop on schedule, forming spicules often indistinguishable from the in vivo counterpart, it is believed the micromeres at the time of their formation are endowed with the determinants necessary and unique for their ontogeny.

The protein component of the spicule has been isolated. Sixty percent of the protein mass is composed of one protein, called the major spicule matrix protein (MSMP). We were interested in cloning the gene for this protein as it represents the terminal differentiation product that characterizes the micromere lineage. Bulk spicule protein was used to raise an antiserum, which in turn was used to screen a phage expression vector cDNA library. This library, and the initial characterization of cDNA clones, were described in last year's Annual Report (see Biology 1984, Abstract No. 33). We have since fully characterized the MSMP gene. It is a single copy gene, and clones have been isolated that contain the entire transcription unit. A single transcription initiation site has been mapped by 5' protection and primer extension analysis. A very large intron (6.2 kb) is found in the 5' coding region 213 bp from the transcription initiation site. Large 5' introns seem to be a prevalent feature of many sea urchin genes and may involve intron-located regulatory elements. Northern gel analysis reveals a single 2.1 kb transcript, which from an initially very low level (perhaps present even in the egg) is induced beginning at 14 hours (blastula stage) to very high amounts in the late embryo. We estimate the prevalence of this transcript in 62-hour embryos at 0.025% of the poly(A)+ RNA, and at 0.75% in cells actively transcribing this gene. Finally, we have determined the entire sequence of the transcript plus some flanking sequence as well. Most interestingly, a large domain encompassing 45% of the protein was found to consist of a series of loosely repeated 13-amino-acid peptides. This sequence is not homologous to any previously reported sequence in the National Biomedical Foundation protein sequence databank. Considering the protein's function, this domain may be involved in embedding the calcite (CaCO₃) crystals, which give the spicule its rigidity.

We are interested in understanding the mechanisms that regulate developmental gene expression, and clearly the MSMP gene will be a powerful marker in future analyses. As described in other reports from this lab, an operational sea urchin embryo transformation system has been established that exhibits proper expression. Experiments will soon be initiated to dissect transcriptional regulation of the MSMP gene in the micromere lineage.

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53. A Study of the Sea Urchin "Bindin" Gene

Boning Gao, Laurie E. Klein

Bindin is a major protein in the acrosome of sea urchin sperm. It binds to the receptor in the egg membrane and renders fertilization species specific. The amino acid sequence of bindin (molecular weight 30,500 daltons) has been determined (Glabe and Vacquier, 1978; Vacquier, unpublished data). We are interested in its gene structure and regulation. Bindin cDNAs have been identified from a random calf thymus DNA-primed and an oligo(dT)-primed sea urchin S. purpuratus testes cDNA library. The probe, made according to the protein sequence, is 17 nucleotides long. Overlapping cDNA clones that cover the entire messenger RNA have been sequenced. There is a 732-nucleotide open reading frame at the 5' end of the cDNA followed by the sequence coding for the mature bindin protein. The sequencing data are consistent with the result we saw from in vitro translation and immunoprecipitations, which show that there is a 60,000 dalton bindin precursor protein. There are three basic amino acids (Arg-Arg-Arg) at the junction of the open reading frame and the N terminus of the mature protein. This suggests that a trypsin-like enzyme is involved in the processing of the precursor protein. Bindin is coded by a single gene as shown by Southern analysis. We are currently studying the gene structure.

Reference:
54. Evolution of the Sea Urchin Bindin Gene  

Joseph Minor, Boning Gao  

Bindin is a sperm acrosomal protein that binds to a receptor on the egg and renders fertilization species specific. Since urchins release their gametes to be fertilized in open water, bindin and its receptor are probably the major species-isolating barrier in urchins. For this reason, work is beginning on the study of the evolution of the bindin gene. The bindin gene of Strongylocentrotus purpuratus has been cloned and is being characterized (see Abstract No. 53). We now plan to study the bindin genes of two congeners, S. drobachiensis and S. franciscanus.

55. Regulation of Spec Gene Expression during Sea Urchin Development  

Craig R. Tomlinson  

A family of mRNAs, termed Spec mRNAs, are synthesized exclusively in the aboral ectoderm of Strongylocentrotus purpuratus embryos (Carpenter et al., 1984). These mRNAs code for acidic low molecular weight proteins belonging to the troponin C superfamily. Spec RNA is present in very low or undetectable amounts in the unfertilized egg and early cleaving embryo and begins to accumulate at blastula stage. The level of Spec RNA increases over 100 fold from early blastula to gastrula stage and declines at the pluteus stage. In situ hybridizations with a Spec-specific probe to sectioned pluteus embryos demonstrated that Spec RNA is localized to the aboral ectoderm.

One of the means by which the Spec genes are regulated may involve the flanking DNA regions and introns. The importance of these genomic regions in the regulation of RNA transcription has been demonstrated in several systems. The 5' flanking DNA regions and the 5' flanking DNA regions together with the 5' introns of the Spec 1b gene will be fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and microinjected into the cytoplasm of unfertilized sea urchin eggs. Assay of the developing embryos for CAT gene expression will allow us to identify any fused DNA sequences that may be regulating the CAT gene. Spec 1b gene transcription and expression will be assayed by in situ hybridization and various other techniques using specific probes. A direct correlation of the expression of CAT with the expression of the endogenous gene would indicate that a regulatory genomic DNA sequence has been isolated.

Reference:  

56. Molecular Immunology of Sea Urchins  

L. Courtney Smith  

Immune systems in vertebrates and invertebrates are capable of distinguishing between self and non-self. Sea urchins also exhibit this attribute. They are able to clear injected bacteria and viruses. They have been shown to reject allografts and are assumed to be able to defend themselves against infection. Sea urchin coelom fluid contains coelomocytes which are considered to be the urchin "immunocytes." However, all molecular mechanisms and most cellular mechanisms underlying invertebrate (and urchin) immunity are unknown.

In this initial investigation of the urchin immune system, we are using probes from the mammalian immune system to analyze the urchin genome and the mRNAs produced by the coelomocytes. With these and various other techniques, we hope to isolate and characterize genes involved in the immune responses in an invertebrate.

57. Isolation of the Ascidian Acetylcholinesterase Gene(s)  

James J. Lee, Thomas H. Mende

We are attempting to clone the acetylcholinesterase (Ace) gene(s) of an ascidian, Cliona intestinalis. This problem is of interest because Ace is expressed only in the muscle cells of the developing ascidian embryo, and is thus an excellent marker for studying the mechanisms that direct the formation of differentiated cell types in these organisms. Previous work indicates that Ace expression, as well as that of other proteins characteristic of embryonic muscle, is controlled transcriptionally. Availability of cloned probes for Ace will allow this possibility to be examined directly. Other uses for genomic clones of Ace include experiments to determine the DNA sequences responsible for tissue-specific expression of this enzyme, and related studies into the possible nature of the elements (protein, RNA) whose presumed
interaction with such sequences results in the histo-
specific occurrence of Ace.

The approach we are using to clone Ace involves
screening genomic blots of ascidian DNA with a cloned
Ace probe from Drosophila provided by P. Spierer. To
establish a range of useful hybridization criteria (i.e.,
before background overwhelms signal), we have used a
sea urchin actin clone that contains sequences repre-
senting the protein-coding portion of the gene. Using
these conditions as a guide, we are now screening
genomic blots with the Drosophila clone. If a signal is
obtained, a Ciona genomic library (prepared in
EMBL-3) will be analyzed for clones containing Ace
sequences. This library will eventually be used to
close other genes of interest, again using heterologous
probes. The conditions established with the actin
hybridizations will be especially useful when searching
for genes whose sequences are expected to be
conserved.

1Marine Biological Laboratory, Woods Hole, MA.

58. Primate DNA Rearrangement
Huay Ru Hwu, John W. Roberts

We have measured the evolutionary changes in
frequency of two interspersed repeated DNA se-
dences, showing that there has been a relatively high
rate of genomic rearrangement during evolution of the
apes. The table below shows the results for the rela-
tive frequencies in the human, chimpanzee, gorilla and
orangutan genomes of the Alu repeat (700,000 copies
in human DNA) and of a high frequency region of the
Kpnl family of repeats (140,000 copies in human DNA).

<table>
<thead>
<tr>
<th>Relative Repeat Frequencies</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Gorilla</th>
<th>Orangutan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu family</td>
<td>1.0</td>
<td>.52</td>
<td>.61</td>
<td>.69</td>
</tr>
<tr>
<td>Kpnl family</td>
<td>1.0</td>
<td>.44</td>
<td>.51</td>
<td>.68</td>
</tr>
</tbody>
</table>

The measurements were made by titration using an
M13 single-stranded probe. The accuracy is sufficient
to reproducibly obtain these relative frequencies.
Thermal stability measurements indicate interspecies
sequence divergencies of about 1.5% between the apes.
Thus sequence divergence is not responsible for the ob-
erved frequency differences. The best interpretation
is that between 50% and 70% of the members of both
repeat families have either been inserted in the human
genoome or deleted from the ape genomes. It appears
that the orangutan has a higher frequency than the
chimpanzee for both of these families, implying that
each of the ape lineages has had a different history of
repeat insertion or deletion since they diverged.

A difference of a factor of two in the Alu
frequency represents the insertion and/or deletion of
at least 400,000 copies, amounting to a total of about
3% of the genome, in the period since these lineages
have diverged (probably about 5-10 million years).
Thus, the number of nucleotides involved in events of
insertion and/or deletion of a single family of repeats
is comparable in magnitude to the number of nucleo-
tides changed as a result of base substitution in total
single copy DNA. The genomic change due to the Kpnl
family is also significant since the Kpnl repeat is about
5 kb long and the inserted and/or deleted region might
often be that large. It is not yet known how many
families of repeats undergo evolutionary insertion
and/or deletion and thus we cannot estimate the total
magnitude of these rearrangement processes but guess
that it is likely to be very large.

59. The Rate of Mutation Differs Between
Systematic Groups
Roy J. Britten

The change of DNA and proteins is of course a
significant part of the evolutionary process and since
their sequences retain quantitative information about
their history, the rates of change are important to
many areas of evolutionary research. A set of about
50 interspecies comparisons of DNA sequences was
assembled from the published literature for cases in
which an estimate of the divergence time of the
lineages can be made. Clear conclusions can be drawn
which are nearly independent of the uncertainties in
the estimates of times of divergence.

First, the interspecies sequence differences of
total single copy DNA (based on thermal stability of
interspecies DNA hybrids) agree closely with primary
sequence differences due to neutral base substitutions.
The implication is that most single copy DNA
sequences have little effect on the phenotype, a result
consistent with the fact that coding regions only make
up a small fraction of the genome of eukaryotes.
The rates of base substitution (for neutral changes or total single copy DNA) are very much larger for sea urchins, some insects, and rodents than they are for primates or birds. The rates deduced from mouse/rat, several sea urchin comparisons and the Hawaiian drosophilids are all similar to each other and average to 0.66% per million years. On the other hand the rate for higher primates is nearly identical to that for birds and equals 0.13% per million years.

The evidence indicates that a retardation occurred in the rate of change of primate DNA sequences about the time that the lineages leading to the lower and higher primates diverged. Since that time the higher primates have apparently consistently maintained the lower rate mentioned above. There is less information from lower primate DNA comparisons but they appear to have exhibited intermediate rates. The implication is that the underlying rate of mutation differs between different systematic groups and is itself influenced by natural selection.

60. Nucleotide Sequence Studies of a Sea Urchin Interspersed Repeat Family

John W. Roberts

The 2101 repeat family in Strongylocentrotus purpuratus consists of approximately 700 copies of a 300 base pair sequence interspersed within single copy DNA. Previous measurements using CS2101, a family member cloned without flanking sequences, suggested large changes in repetition frequency during recent sea urchin evolution. The S. franciscanus genome, for example, has only about fifty 2101 repeat family members; in spite of this difference in copy number, the nucleotide sequences of family members appear to have been conserved to a greater extent than total single copy DNA. Interspecies differences in repetition frequency are expected for transposable genetic elements, since rates of amplification, dispersal, and deletion may differ after species divergence.

The 283 nucleotide sequence of the originally cloned family member, CS2101, has been determined. To further investigate the evolutionary behavior of the 2101 repeat family, five family members along with associated flanking sequences have been cloned from the S. purpuratus genome. Nucleotide sequence analysis of the genomic clones is in progress to look for short target site sequence duplications and other features characteristic of transposable elements. The sequence of an approximately 200-nucleotide-long segment of one genomic clone has been determined. Comparison of this sequence with that of CS2101 confirms the level of intraspecies similarity of 2101 family members previously measured by thermal denaturation. In separate experiments, specific flanking sequence probes will be used in screening assays of individual genome libraries to test for 2101 repeat mobility within the S. purpuratus genome.

61. Expression of Retrovirus-like Genomes in Early Mouse Embryos

Lajos Piko, Ken D. Taylor

Early embryos of all mouse strains examined, including feral mice, regularly express two morphological subtypes of intracisternal A-type particles which appear at specific stages of development. One subtype of particles (referred to as IAP), which morphologically resembles the particles occurring in many mouse tumors, is seen only in oocytes and late cleavage-stage embryos. We have shown recently that the IAPs occurring in early embryos and in tumor cells are, in fact, genetically identical (Piko et al., 1984). The role of the IAP genomes in embryo development is not known but there is evidence suggesting that IAP-related products contribute to the differentiation of the cell surface in early embryos.

The other subtype of particles, termed epsilon particles, is particularly intriguing for several reasons. 1) Epsilon particles have not been observed in cells other than early embryos. 2) These particles are formed in very large numbers ($10^5$ to $10^6$ particles per embryo) at the two-cell stage but disappear after two to three cell divisions. 3) Epsilon particles appear to be the precursors of a nucleoprotein structure called "crystalloid" which persists in the cytoplasm until the time of implantation.

To analyze some of these problems, we have recently isolated a cDNA library derived from poly(A)$^+$ RNA of late two-cell embryos. This library will be screened with labeled cDNA from embryos at different stages of development. Based on their abundance at the two-cell stage and other expected properties, cDNA clones representing epsilon particle sequences should be identifiable. This approach should provide information on the pattern of expression of some other
gene products as well.

References:

1 Veterans Administration Medical Center, Sepulveda, CA.

PUBLICATIONS


Britten, R. J. (1985) Rates of DNA sequence evolution differ between taxonomic groups. Submitted for publication.


Professor: William J. Dreyer
Research Fellow: David B. Teplow
Staff Associate: Janet M. Roman

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Summary: Bill Dreyer and Janet Roman-Dreyer are currently working in Tübingen, Germany, in collaboration with scientists at the Max-Planck-Institut für Entwicklungsbiologie (Developmental Biology). Their aim is to combine the resources and skills available at the Max-Planck-Institut with those now available at Caltech in a study of molecules (and genes) that control cell recognition processes during embryological development. We have long been fascinated by the descriptive biology that clearly indicates that embryonic cells and nerve fibers display a high degree of specificity during embryogenesis and regeneration (see for example, Sperry, 1963). Nevertheless, we could only speculate on the possible nature of genes and molecules that control these biological phenomena (Dreyer et al., 1967; Hood et al., 1977; Dreyer, 1984; Dreyer and Roman, 1984). The reason
that no definitive experiments explaining the genetic and molecular bases of cell-cell interaction during embryogenesis have yet been carried out is that the methods and instruments required have not been available. This fact was one of the strong motivations that led to the development of the Caltech Microchemical Facility in collaboration with Prof. Lee Hood and his group (Hunkapiller et al., 1984). We have used the facility in a successful feasibility project to demonstrate that scarce cell-surface proteins involved in cell-cell recognition processes of blood cells can be isolated, sequenced (Springer et al., 1983), and the genes cloned (see Abstract No. 63). It now seems possible to begin a serious study of molecules and genes involved in cell-cell recognition during embryogenesis. The research groups of U. Schwarz, F. Bonhoeffer, and A. Gierer are all active in the study of nerve fiber specificity in the developing retina. It seems likely that fruitful collaborations will be developed during our stay in Tübingen.

References:

62. The Novel Leukocyte Adhesion Family
LFA-1/Mac-1 Is Related to Leukocyte Interferon

David B. Teplow, Timothy A. Springer
dr, William J. Dreyer

Cell-surface adherence reactions are fundamental to the biology of lymphocytes, monocytes and granulocytes. The lymphocyte function-associated 1 (LFA-1) and macrophage 1 (Mac-1) glycoproteins mediate differing types of adhesion reactions on these cells. LFA-1 participates in T-lymphocyte and natural killer cell adhesion to target cells (Davignon et al., 1981; Pierres et al., 1982; Springer et al., 1982), whereas the Mac-1 antigen is identical to the complement receptor type 3, which mediates adhesion of monocytes and granulocytes to C3bi-sensitized particles (Beller et al., 1982). Deficiency of these proteins, in a heritable disease, results in multiple adhesion-related leukocyte defects (Anderson et al., 1984; Springer et al., 1984). LFA-1 and Mac-1 resemble one another in overall structure, having α-subunits of relative molecular mass (Mr) 180,000 and 170,000, respectively, which are non-covalently associated with β-subunits of Mr 95,000 in α1β1 complexes. Peptide mapping and immunological cross-reactivity have shown that the β-subunits are highly related if not identical, but have revealed no similarities between the α-subunits (Trowbridge and Omary, 1981; Kürzinger and Springer, 1982; Kürzinger et al., 1982; Sanchez-Madrid et al., 1983).

Nonetheless, the shared β-subunit suggested that LFA-1 and Mac-1 might be members of a protein family containing diversified but evolutionarily related α-subunits.

We have examined the structures of the α-subunits by N-terminal amino-acid sequencing. Sequence homology shows that the α-subunits are members of a novel leukocyte adhesion protein family, and suggests that their evolution occurred by gene duplication (Springer et al., 1984).

A search for similarities to previously sequenced proteins reveals a further unexpected homology between LFA-1 and leukocyte (α) interferons (Springer et al., 1985). Our findings suggest that the LFA-1/Mac-1 α-subunit family and the interferon family arose from a single ancestral gene and can be considered as a superfamily. This is remarkable for several reasons. First, LFA-1 and Mac-1 are surface receptors, whereas the interferons are secreted mediators. Second, there are striking differences in size. The LFA-1 and Mac-1 α-polypeptide chains (Mr 180,000 and 170,000) are roughly 10 times longer than those of the interferons Mr 20,000). Despite the difference in size, the N-terminal sequence of LFA-1 aligns only six residues away from the N terminus of IFN-α. A common evolutionary mechanism for obtaining large proteins is tandem duplication and joining of domains. Further sequencing of LFA-1 is
needed to determine whether it contains the homologue of an entire interferon domain and whether it contains duplications of these domains.

References:

1 Dana-Farber Cancer Institute, Boston, MA.

63. Cloning of Murine Mac-1-specific DNA Sequences Directly from a Genomic Library

Janet M. Roman, David B. Teplow, Timothy A. Springer1, William J. Dreyer

The lymphoid cell-surface protein LFA-1 and the macrophage cell-surface protein Mac-1 are differentially expressed during development of these cell lines from common stem cells. These molecules are structurally similar and protein sequence analyses suggest they evolved from a common ancestral gene (Springer et al., 1985), yet they have functionally discrete roles in cell-cell interactions of the immune system (see Abstract No. 62).

The LFA-1/Mac-1 cell-surface proteins provide an excellent system for the study of the control and structure of molecules involved in cell interactions. We wish to answer such questions as: 1) How large is the LFA-1/Mac-1 family? 2) Are related molecules expressed on other cell types? 3) Are genes coding for members of this family arranged in tandem with control features similar to those of their immunoglobulin, a-globin or other known multigene families? 4) What mechanisms control lineage-specific expression of these proteins?

To answer such questions, we have begun the cloning of their genes. The most commonly used approaches for cloning specific genes from cDNA libraries are quite disadvantageous when dealing with genes that are both rare-message genes and are quite large (the a chain of Mac-1 is a protein of molecular weight 170,000). Furthermore, our probes consisted of synthetic oligonucleotides corresponding to the N terminus of the protein, a region rarely represented in a cDNA copy of a large message. To circumvent this problem, we have designed and had constructed synthetic oligonucleotide probes complementary to two overlapping regions of the Mac-1 gene (Biology 1983, Abstract No. 27). Using these probes under carefully selected conditions of stringency (Conner et al., 1983), we screened a Charon 4A library made from a partial EcoRI digest of BALB/c spleen DNA (generously provided by Dr. Roger Perlmutter in Prof. Hood’s lab). Approximately 1.3 million plaques were screened with each of two pools of synthetic cDNA eicosamer libraries. Sixty positive plaques were selected, purified, and then rescreened with each of the two pools of overlapping eicosamers. Of the original 60 clones, two were positive with the overlapping probes. These two clones are identical. The sequence of portions of these clones obtained by the Maxam-Gilbert protocol has revealed DNA sequence (beyond the bases corresponding to the synthetic probes used for isolation) which matches codons expected for the known protein sequence of the N-terminal region of Mac-1.

The successful cloning of this gene leads the way to analysis of its structure and its control regions, perhaps clues to its regulation during differentiation, and the study of other LFA-1/Mac-1 family members.

References:

1 Dana-Farber Cancer Institute, Boston, MA.

PUBLICATIONS


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Summary: Our laboratory is interested in a variety of areas including the molecular biology of the immune response, of certain growth factors and their receptors, and of myelin basic protein, as well as the development of chemistries and instrumentation for the analyses and synthesis of genes and proteins. We employ a wide variety of techniques ranging from immunology to molecular biology to sophisticated protein and DNA chemistries.
The vertebrate immune response is a complex response to foreign macromolecules (antigens) that employs three major families of receptors: antibodies, T-cell receptors and cell-surface proteins encoded by the major histocompatibility complex (MHC). We study each of these receptor gene families. In the past year, we have accomplished several major goals. First, the structures and organizations of the α and β T-cell receptor genes have been largely delineated. Second, the rearrangements, diversification and ontological expression of the T-cell receptor genes have been generally delineated. Third, the germline diversity of one subfamily of human antibody V\textsubscript{H} gene segments has been found to be surprisingly large. Fourth, tools for looking at the function and regulation of class I and class II MHC genes are being exploited. Fifth, functional implications of the interactions among MHC and T-cell receptor genes are being studied. Sixth, sequence data from each of these three gene families have established that all are members of the immunoglobulin gene superfamily and hence their mechanisms for functional interactions probably employ similar strategies. Future studies are concentrating in how these gene products function and how they are regulated.

We are also interested in several growth factors and their receptors, interleukin-3 (IL3) and platelet-derived growth factor. Efforts are under way here to clone both receptors. Perhaps more important, we have applied the powerful protein synthesis techniques to successfully synthesize the entire 140-residue IL3 molecule and several analogues. Ultimately we wish to understand how IL3 and its receptor carry out their diverse array of functions.

The gene encoding myelin basic protein (MBP) has been cloned in mice and efforts are under way to clone it from human DNA. This mouse gene is large, ~34 kb, and it undergoes alternative patterns of RNA splicing to generate at least four distinct gene products, whose functions are unknown. We have also determined that the shiverer mice lack a complete gene for MBP. Experiments are now under way attempting to construct transgenic shiverer mice with the wild-type gene for MBP. We would like to understand what the four MPBs do and how the MBP gene is regulated.

A major effort in our laboratory has been centered around the microchemical facility—an effort to develop new chemistries and instrumentation for the synthesis and analysis of proteins and genes. Over the past several years, we have developed the gas phase protein sequenator, a DNA synthesizer and a peptide synthesizer. We are now in the process of developing a DNA sequenator as well as a second generation protein sequenator that will allow us to analyze proteins at the 10-100 femtomole levels. We are also developing new protein separation techniques for the isolation of very small amounts (femto- to picomole quantities) of material. In addition, we are developing a variety of new DNA chemistries. There have been several spectacular applications of the automated peptide synthesizer to the analysis of structure-function relationships, including the complete synthesis of the IL3 protein and the analysis of the epitopes of human hepatitis B virus. The microchemical facility has opened up a myriad of new and exciting research opportunities in a variety of different areas.

64. Analysis of T-Cell Receptor β-Chain Genes in T Cells of Defined Antigen Specificity and MHC Restriction

Joan Goverman, Karyl Minard, Nilabh Shastri, Tim Hunkapiller

Antigen receptors on both B and T cells are composed of two chains, the light and heavy chains in B-cell receptors and α and β chains in T-cell receptors. Because B and T cells recognize a large array of foreign antigens, extensive diversity in their receptor repertoire is required. Both cell types utilize similar strategies of gene organization and genetic diversification to achieve diversity in their receptors. However, antigen recognition by T cells differs in several respects from antigen recognition by B cells. Instead of binding free antigen, T cells recognize antigen only when it is presented on the cell surface in association with MHC molecules. This recognition is MHC-restricted in that a T cell will recognize antigen only when it is associated with particular MHC alleles, usually those expressed in the thymus in which the T cell has matured. We have studied the receptor genes of functional T-cell lines that recognize either different or related antigenic determinants and MHC molecules in order to learn how these different properties of recognition can be accounted for in the molecular structure of the receptor.

We characterized all of the β-chain rearrangements that occurred in a helper T cell specific for the
antigen hen egg-white lysozyme and restricted by the I-A\(^B\) molecule. This cell line contained two non-productive \(\alpha\)-chain rearrangements and one productive rearrangement joining a \(V_\beta\) gene segment to \(D_\beta-J_\beta-C_\beta\) sequences. These observations demonstrated that the principle of allelic exclusion established for B cells, which predicts only one functional rearrangement will be found for each receptor chain, is followed in this T-cell line. Our analysis of the productive \(\alpha\) chain showed that the \(V_\beta\) gene segment expressed in this lysozyme-specific line is the same as that expressed by a T cell specific for cytochrome c and the I-E\(^k\) MHC molecule. This result shows that there is no simple correlation between \(V_\beta\) gene segments and antigen specificity on MHC restriction. By analyzing other cytochrome-specific T cells, we found that T-cell receptors that recognize the same MHC molecule and very similar antigens can express completely different \(\beta\) chains. Thus, the structural features that impart specificity for both antigen and MHC determinants to the T-cell receptor are likely to result from interactions between the \(\alpha\) and \(\beta\) chains and not derive from either chain individually. Analysis of our data compared to other published sequences of \(\beta\) chains shows that the \(D_\beta\) gene segments can be translated in any reading frame providing the \(J_\beta\) gene segment is in frame, unlike \(D\) gene segments in heavy chains, which usually are translated in only one reading frame. This allows T cells to generate diversity in their receptors in a way not frequently observed in B cells.

References

65. Expression of Cloned T-Cell Antigen Receptor Genes
Joan Goverman, Karyl Minard

There are three recognized subsets of T lymphocytes: cytotoxic T cells, which kill target cells expressing antigen on the cell surface, and helper and suppressor T cells, which stimulate or suppress immune responses, respectively. The response of each of these classes of T cells involves recognition of antigen in association with particular MHC molecules which then signals the T cell to proliferate and carry out its effector function. In addition to the isolation of genes encoding the T-cell receptor for antigen, recent progress in immunology has identified some of the components involved in transmitting this signal as well as accessory molecules that may play a role in antigen recognition. We are interested in understanding the mechanisms that control these processes and in identifying the molecules responsible for carrying out particular effector functions. We have cloned T-cell antigen receptor genes from helper T cells of defined antigen specificity and MHC restriction, and we are employing DNA-mediated gene transfer to introduce these genes into other functional T cells. We will determine if a new antigenic specificity can be introduced into T cells as a result of expressing the cloned receptor genes and what role accessory molecules may play in facilitating recognition. We also will determine if the expression of receptor genes from a helper T cell in a cytotoxic T cell will allow this cell to mediate cytotoxicity with a specificity for novel antigenic and MHC determinants.

66. The Murine T-Cell Receptor Employs a Limited Repertoire of Expressed \(V_\beta\) Gene Segments
Richard K. Barth, Byung S. Kim, Nancy Lan, Tim Hunkapiller, Nancy Sobieck, Astar Winoto, Howard Gershenfeld\(^1\), Craig Okada\(^1\), Dan Hunsburg\(^2\), Irving Weissman\(^1\)

The sequences of eight cDNA clones containing \(V_\beta\) genes of the mouse T-cell receptor have been determined and analyzed with seven \(V_\beta\) genes reported previously. Among the 15 \(V_\beta\) genes, only ten different \(V_\beta\) gene segments are employed. This repeated occurrence of \(V_\beta\) gene segments implies that the expressed repertoire of \(V_\beta\) gene segments is small. A statistical analysis of the frequency of finding identical \(V_\beta\) gene segments with the sample analyzed suggests that the total number of expressed \(V_\beta\) gene segments is 21 or less. This estimate is much less than the number of immunoglobulin heavy or light chain \(V\) gene segments. DNA blotting studies also suggest that the \(V_\beta\) gene segment repertoire is small. This result, in conjunction with the apparently low frequency of somatic hypermutation, implies that \(\beta\)-chain diversification is concentrated in the \(V_\beta-D_\beta-J_\beta\) junctions.

DNA blot analysis of mouse genomic DNA with probes specific for each of the ten different \(V_\beta\) gene segments indicates that they are organized into eight distinct \(V_\beta\) subfamilies, designated \(V_{\beta1-V_{\beta8}}\). Six of these subfamilies have only a single gene segment-
member, unlike immunoglobulin V subfamilies which have 100-300 members.

Analyses of the distributions of variable amino acid residues, $\beta$-pleated sheet-forming potential and relative hydrophobicity of $V_\beta$ regions indicate that they are structurally very similar to their immunoglobulin counterparts. In addition, $V_\beta$ regions conserve most of the same amino acids thought to be necessary for both $V_H$ and $V_L$ inter- and intrachain interactions. These observations suggest that T-cell receptor and immunoglobulin variable domains fold into similar tertiary structures. This prediction, in conjunction with the absence of any correlation of particular $V_\beta$ gene segments with individual antigen or MHC specificities, suggests that the $\alpha$ and $\beta$ chains together play a critical role in generating the binding site for antigen plus MHC.

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67. Analysis of the T-Cell Receptor $\beta$-Chain Genes from Lysozyme-specific T-Cell Hybridomas

Joan A. Kobori, Nilabh Shastri

We have analyzed $\beta$-chain gene rearrangements in eight T-cell hybridomas specific for the T11 peptide (residues 74-96) of hen egg lysozyme. Three of these T-cell hybridomas recognize the peptide antigen in the context of the MHC I-A$k$ molecule, whereas the other five T-cell hybridomas are I-E$k$ restricted. The I-A$k$-restricted cells recognize determinants contained within the amino-terminal residues 74-86 while the I-E$k$-restricted cells recognize determinants of the C-terminal residues 85-96. These observations suggest that specific interactions between certain residues of the antigen and the restricting MHC molecule are important for determinant selection.

Using specific hybridization probes for the $C_{\beta 1}$ gene and the $J_{\beta 2}$, $D_{\beta 1}$, and $D_{\beta 2}$ gene segments, we identified the productive lysozyme-specific $\beta$-chain rearrangement in each T-cell hybridoma by DNA blotting techniques. Size-selected limited genomic DNA libraries were constructed using $\lambda$ phage vectors to isolate the productive $\beta$ chain rearrangements. With the use of available oligonucleotide probes specific for each J gene segment as well as cloned $V_\beta$ gene segment probes, we could identify five distinct $V_\beta$ and six distinct $J_\beta$ gene segments used by these T cells. Hence, the T cells we analyzed are not derived from a single set of rearranged receptor genes that have, for example, undergone somatic hypermutation events. We are currently sequencing the V-D-J gene segments to analyze the detailed structure of these T11 peptide-specific $\beta$ chains. Our results indicate that T-cell responses to single determinants such as peptide antigens are made up of many independent clonotypes.

68. Is Somatic Hypermutation Employed by T-Cell Receptor $V_\beta$ Genes As One of the Mechanisms for Generating Diversity?

Nancy Lan

The structure of the $V_\beta$ genes of the mouse T-cell receptor has been extensively studied recently. It is evident from these studies that $V_\beta$ genes employ mechanisms for generating diversity that are both similar to and different from the mechanisms of their counterpart immunoglobulin V genes. The following mechanisms are shared by both V genes: 1) multiplicity of germline V, D and J gene segments; 2) combinatorial joining of these gene segments; 3) flexibility in the sites at which they are joined; and 4) N-region diversity, or the addition of random nucleotides to either side of the D gene segment in the assembly of the V gene. Two major different mechanisms are observed. First, the $D_\beta$ gene segments of $V_\beta$ genes may join to the $V_\beta$ gene segments in all three translational reading frames, whereas $D_H$ gene segments generally join their D gene segments in one translational frame. Second, somatic hypermutation, which can generate single nucleotide substitutions throughout the entire V gene, is often employed by immunoglobulin $V_H$ genes but appears to be rarely employed by $V_\beta$ genes, if at all.

The limited studies conducted thus far are inconclusive as to whether somatic hypermutation occurs in the $V_\beta$ genes. Results of two studies of two $V_\beta$ gene segments in two T cell lines failed to demonstrate hypermutation (Chien et al., 1984; Goverman et al., 1985), and in other studies (Barth et al., 1985), nucleotide substitutions were detected among several $V_\beta$ genes, but those could have been due to strain polymorphism. The objective of the current study is to determine whether somatic hypermutation is employed by T-cell receptor $V_\beta$ genes. In a study of
a panel of T-cell hybridomas, which were obtained from several MHC-congenic strains of mice [B10.A, B10.S(9R) and B10SM] immunized against peptides of cytochrome c, we found that three of these hybridomas appear to have the same rearranged \( V_\beta \) gene (\( V_\beta_3 \)) by Southern blotting analyses.

These \( V_\beta \) genes have been cloned and will be sequenced. The sequence data should provide information on the frequency of hypermutation employed in T-cell receptor \( V_\beta \) genes.

References:

69. Genes Encoding the V Regions of Alloreactive T-Cell Receptors

Nancy A. Costlow

The antigen receptor on T cells is composed of two chains, \( \alpha \) and \( \beta \). Each chain consists of both variable (V) and constant (C) regions, as well as short diversity (D) and joining (J) segments. Recent studies (Arden et al., 1985; Barth et al., 1985) have indicated that there may be only a relatively small number of germline \( V_\beta \) genes and as many as 40 or more \( V_\alpha \) genes. The diversity of the T-cell response is partially generated through the use of these different germline V genes. In addition, the combinatorial joining of different V, D, and J genes and somatic mutations at the D segment boundaries contribute to T-cell diversity.

We are examining the genes encoding T-cell receptors that are reactive with a number of very closely related antigens. These antigens, the MHC class I protein K\(^b\) and mutations thereof (Nairn et al., 1980), differ from each other by only 1-3 amino acids. In this study, we hope to determine by which mechanisms T cells are able to distinguish between such closely related antigens.

References:

70. Diversity and Structure of Genes of the T-Cell Antigen Receptor \( \alpha \) Family

Bernhard Arden, Joan L. Klotz, Gerald Siu

We isolated 19 cDNA clones encoding the \( \alpha \) chain of the T-cell antigen receptor from a thymic library using an \( \alpha \) constant region probe. Fifteen of these clones contain partial or complete variable (\( V_\alpha \)) genes. They can be divided into ten different subfamilies, members of each subfamily being 80% or more homologous to one another at the DNA level. Complete \( V_\alpha \) genes from seven different subfamilies hybridize to more than 40 germline \( V_\alpha \) gene segments in Southern blot analyses, each subfamily ranging in size from 1-10 members.

Of 19 joining (\( J_\alpha \)) sequences examined, 18 appear to be encoded by distinct gene segments. Since we found only one repeat, the repertoire of \( J_\alpha \) gene segments is probably considerably larger than 18, much larger than those seen in the immunoglobulin or T-cell receptor \( \alpha \)-gene families. The boundaries of the \( J_\alpha \) segments were determined by comparison with their germline genes (Winoto et al., 1985). The large \( J_\alpha \) repertoire along with other mechanisms increase the diversity at the carboxy-terminal end of the \( V_\alpha \) region.

One cDNA clone appears to be either a germline \( J_\alpha \) transcript or a transcript from an incomplete D-J rearrangement. It contains a \( J_\alpha \) sequence and a 5' recognition sequence for DNA rearrangement with a 12 bp spacer. Since the germline sequence of this \( J_\alpha \) sequence has not yet been determined, we do not know whether the recognition sequence lies to the 5' side of a diversity (\( D_\alpha \)) or a \( J_\alpha \) gene segment.

The \( V_\alpha \) gene segments show the two classically-defined and possibly additional areas of hypervariability. The \( V_\alpha \) and \( V_\beta \) regions share 18 highly conserved residues with the \( V_H \) and \( V_L \) regions of immunoglobulins, all of which are important for stabilization of the immunoglobulin fold. The secondary structures of the \( \alpha \) polypeptides appear to resemble those of their \( \beta \) and immunoglobulin counterparts, suggesting that the variable domains of immunoglobulins and T-cell antigen receptors are similar in structure to one another (Arden et al., 1985).

References:
71. Genomic Organization of the Genes Encoding the Mouse T-Cell Receptor α Chain

Astar Winoto, Shelley E. Mjolsness

Using a T cell minus B cell subtractive probe, we have previously isolated 64 T cell-specific cDNA clones from a helper T cell (TH) 1.9.2 cDNA library that we constructed in λgt10 vector. We have analyzed every clone on a Southern genomic blot to look for DNA rearrangements in the cytochrome c/1-Eα-Eβ-specific TH 1.9.2 hybridoma. One of the 64 clones is the T-cell receptor α chain, which rearranges in the TH 1.9.2. The other 63 clones, however, have no detectable DNA rearrangement in the TH 1.9.2 cell line. One of them was later shown to be an α chain constant region-specific cDNA by hybridization to an oligonucleotide made according to the published α cDNA sequence (Chien et al., 1984; Saito et al., 1984).

The explanation for our inability to detect rearrangement in the T-cell receptor α gene using a Ca probe became apparent when we analyzed the genomic organization of the α chain.

We have isolated clones encoding a germline variable (V) gene segment and cosmid clones spanning 120 kb of DNA encoding the Jα gene segments and the Ca gene. Analysis of these clones shows that the DNA recognition sequence for α-gene rearrangement is similar to its β-gene counterpart. In contrast to the general organization of the Jβ, Jγ, JH and JK gene segments, we showed that the Jα gene segments are not closely clustered. The 18 Jα gene segments analyzed were spread over 60 kb of DNA and lie as far as 63 kb 5' to the Ca gene (Figure 1) (Winoto et al., 1985).

The sequence and organization of the Jα gene segments, as well as observation by others (B. Arden, J. Klotz, G. Siu) that 18 Jα gene segments from 19 cDNA clones are distinct, suggest that diversity plays an important role in forming the T-cell receptor-binding pocket. Thus, Jα gene segments may play a central role in generating the T-cell receptor diversity and may be functionally selected to be diverse in length and in sequence, as well as large in number.

References:

Figure 1. A restriction map of the mouse cosmid cluster containing the Jα gene segments and the Ca gene. Boxes indicate restriction fragments that hybridize to the 500 bp Sau3A fragment Ca probe from A10 cDNA clone or to the synthetic oligonucleotide Jα probes. Filled boxes indicate the Jα gene segments that have been sequenced (Winoto et al., 1985). A (+) between two restriction sites indicates the presence of unassigned sites in this region. The Jα gene segments lie to the 5' side of the Ca gene, whose direction of transcription is to the right. The Jα gene segments are named according to the cDNA clone from which they originated.
72. Isolation of T-Suppressor-specific Genes

Hilde Cheroutre, Judy Kapp

The different pathways by which T-suppressor cells (T_s) and their factors act seems to be complicated and currently molecular the knowledge of the T_s system is incomplete and often controversial. Identification and characterization of purified suppressor molecules that bind antigen, as well as isolation of their genes, should clarify the mechanism by which those T-suppressor cells function and should suggest new areas of theory and experiments.

In our approach to this problem, we are attempting to identify T-suppressor-specific genes from a T-suppressor hybridoma (372 D_6.s) specific for the polymer L-glutamic acid^L^-L-alanine^30^-L-thyrosine^10 (GAT) by screening a cDNA library with subtracted-specific probes. GAT-specific T-suppressor cells were fused with the hypoxanthine-guanine phosphoribosyl transferase-deficient AKR thymoma BW5147. Prior to RNA isolation, those cells were induced with immune interferon and interleukin-2. Double-stranded cDNA was made from the poly(A)^+ membrane-bound RNA fraction. EcoRI linkers were ligated with the blunt-ended cDNA. After an EcoRI digest, the cDNA was sized on a BioGel A50 column. Different sized pools were ligated into EcoRI-cut bacteriophage λgt10 vector arms. We are now in the process of screening a part of the cDNA library with insert sizes between 1500 bp and 3000 bp. cDNA synthesized on the same poly(A)^+ RNA fraction as the cDNA library and subtracted against both B-cell mRNA and BW5147 mRNA was used as probe for the initial screening. One hundred and twenty-five clones were selected for a differential screening with cDNA of BW5147 mRNA and cDNA of 372 D_6.s mRNA as probes. The clones hybridizing only with 372 D_6.s cDNA should derive from the suppressor cell. These clones will be analyzed further. We will eventually look for rearrangements and screen the clones with probes subtracted against T-helper cell mRNA and cytotoxic T-cell mRNA as well as against mRNA of suppressor T cells with another antigen specificity.

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73. Ontogeny of T-Cell Receptor Gene Rearrangement and Expression

Regina Haars, Mitchell Kronenberg, Frances Owen

Rearrangement and transcription of immunoglobulin genes during fetal development is an ordered process. We have investigated whether the T-cell receptor α-, β- and γ-chain genes are rearranged and expressed in a similar fashion by hybridization of cloned T-cell receptor probes to nucleic acids from fetal lymphoid tissues and hybridomas derived thereof. Several conclusions can be drawn from this work. First, Northern blot analysis shows no transcription of the α-, β- or γ-chain genes in fetal liver day 12-18 as well as neonate. This result indicates that these genes are not expressed at all, or that prethymic T cells expressing α-, β- and γ-chain genes represent only a very small fraction of the cells in the fetal liver. Second, α-, β- and γ-chain transcripts can be detected in the fetal thymus. At day 14, the earliest day tested for all three chains, the γ chain is transcribed at high levels, reaching maximum expression at day 15 followed by a slow decline until day 18 and a great decrease at birth. Expression of the 1.0 kb β-chain RNA is detected at day 14, whereas the 1.3 kb β-chain gene transcript derived from a fully assembled V-D-J rearrangement appears only at day 16. Furthermore, it is observed that the C_β2 gene cluster is more frequently transcribed than the C_β1 gene cluster. A 1.3 kb α-chain gene transcript is present at day 16 as well as small amounts of a 1.6 kb transcript that is first strongly detected at day 17. We conclude that there is an ordered expression of T-cell receptor genes during fetal development whereby γ-chain gene transcription at day 14 precedes a functional β-chain gene transcript (day 16) by approximately two days, followed one day later by a functional α-chain gene transcript (day 17). Third, analysis of whole fetal liver and thymus tissues for β- and γ-chain gene rearrangements shows no rearrangement for either chain in the fetal liver. However, β and γ rearrangements can be detected in the fetal thymus. Discrete rearranged fragments are visible by day 14 using C_β3 as a probe. Some of these rearranged fragments are consistent with D_β1-J_β1 rearrangements, although some of them are inconsistent with this possibility and remain to be explained. Beta gene rearrangements are detected as early as day 13 prior to the presence of a
1.0 kb transcript at day 14. We conclude that at least for the \( \beta \) chain, \( D_\beta-J_\beta \) rearrangements precede \( V_\beta-D_\beta-J_\beta \) rearrangements. Gamma rearrangement is near adult level at day 15 (earliest day tested) and most likely rearranges before the \( \beta \) chain, which has to be earlier than day 13. Alpha gene rearrangements are difficult to detect, but we assume from the transcriptional data that the \( \alpha \) chain is rearranged last. Fourth, analysis of fetal thymus hybridomas for \( \alpha \) and \( \gamma \) rearrangements supports the idea that the \( \gamma \) chain is rearranged and expressed first. Together the results indicate that T-cell receptor \( \alpha \), \( \beta \) and \( \gamma \)-chain gene rearrangements and expression are not random, but are instead a regulated process that proceeds in an ordered fashion.

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74. Rearrangement of T-Cell Antigen Receptor Genes in Heterogeneous Lymphoid Populations

Mitchell Kronenberg, Regina Haars, Max Cooper*, Michael Gallatin

Expression of both immunoglobulin and T-cell antigen receptor genes requires gene rearrangement. In addition, immunoglobulin gene expression exhibits allelic exclusion, meaning that only one of the two possible homologues encoding an immunoglobulin polypeptide is actually productively rearranged and expressed. Recent evidence suggests that the \( \beta \) chain of the T-cell antigen receptor exhibits allelic exclusion as well (Goverman et al., 1985; Kronenberg et al., 1985; Malissen et al., 1985). We recently reported that 37 T cells, including 23 functional T-cell clones and 14 T-cell tumors, had at least 97 different \( \beta \)-chain gene rearrangements (Kronenberg et al., 1985). The allelic exclusion hypothesis predicts that no more than 37 of these rearrangements (1 per cell) are productive. To demonstrate that this large amount of rearrangement is not an artifact resulting from long-term culture of cloned T-cell lines, we performed Southern blots on DNA prepared from heterogeneous T-lymphocyte populations obtained directly from peripheral blood or lymphoid organs. Because each lymphocyte clone will have a unique rearranged restriction fragment, particular rearranged fragments cannot be detected. The loss of signal from the germline restriction fragment due to \( \beta \)-gene rearrangement can, however, be quantitated. We determined that purified T-cell populations (Leu-3+) derived from peripheral blood of humans or the total thymocytes from adult mice had a \( \geq 90\% \) loss of the germline signal using probes that detect \( J_\beta \) gene segment rearrangement. This demonstrates that, in general, rearrangements occur on both homologues and confirms the earlier results with long-term cultured T cells. Faint rearranged \( \beta \) gene restriction fragments could sometimes be detected; most of these are derived from partial \( (D_\beta-J_\beta) \) rearrangements. Thymocytes fractionated on the basis of their ability to bind the lectin PNA or their expression of several different cell-surface markers all had a similar high level of \( \beta \)-gene rearrangement, indicating that most of the T cells in this organ, despite their inability to respond to antigen or mitogen, have fully rearranged \( \beta \) chain.

Natural killer (NK) cells are non-T, non-B lymphoid cells that may be important in the immune response to tumors. Cells expressing NK surface markers that were selected by fluorescence-activated cell sorting from the peripheral blood of normal human donors displayed significant \( \beta \)-gene rearrangement. More than 50% of the chromosomes in these populations had been rearranged. The functional importance of these rearrangements for NK activity remains to be determined.

References:

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75. The Repertoire of \( V_\beta \) Gene Segments Expressed in Human T Cells

Patrick J. Concannon, Lulu Pickering

The \( \beta \) chain of the murine T-cell receptor differs from the model provided by immunoglobulins in that substantially less of its diversity is directly encoded in the germline. The repertoire of \( V_\beta \) gene segments in the mouse is small, probably less than 30, and the...
76. Analysis of a Human $V_\beta$ Gene Subfamily

Gerald Siu, Erich C. Strauss

We are studying one $V_\beta$ gene subfamily, called the $V_{\beta M3}$ subfamily, of the human T-cell $\beta$ gene family. Using a $V_{\beta M3}$-specific probe, cosmid and lambda clones containing the five members of the family have been isolated. One member of this family, $V_{\beta M3-1}$, encodes the $V_\beta$ region of the T-cell receptor on the MOLT-3 tumor (Siu et al., 1984). The basic structure of the $V_\beta$ gene segment is very similar to the immunoglobulin $V$ gene segments. The first exon is approximately 49 bp long and contains most of the leader region; the second exon is 295 bp long and contains 14 bp of the leader region and 281 bp of the variable region sequence. There is an intron of about 100 bp that splits the leader at codon position -5. The 3' flanking region contains the recognition signals for V-D rearrangements; a conserved heptamer and nonamer sequences separated by a nonconserved 23 bp spacer. Analysis of the coding regions reveals that the members of this family are mutating at a rate comparable to that of most eukaryotic genes and therefore much slower than immunoglobulin $V$ gene segments. Two of the five $V_\beta$ gene segments in this family have in-frame stop codons and therefore are pseudogenes. Thus, the $\beta$ gene family, like the immunoglobulin gene families, has pseudogenes. The germline clones containing the members of this family also contain large amounts of flanking region sequence: one cosmid contains the $V_{\beta M3-1}$ and $V_{\beta M3-2}$ gene segments, linked 3 kb apart and 38 kb of 5' and 3' flanking region; two additional cosmids contain the $V_{\beta M3-4}$ gene segment and 40 kb of flanking sequence; and two lambda clones have 9 kb and 43 kb surrounding the $V_{\beta M3-0}$ and $V_{\beta M3-3}$ gene segments, respectively. No additional $V_\beta$ gene segments can be detected in these flanking regions. Thus, the spacing distance between germline $V_\beta$ gene segments ranges from 3 kb to greater than 40 kb. This range of spacing distances is very similar to what is observed in immunoglobulin $V$ gene families. These similarities between the immunoglobulin $V$ gene segments and the $V_\beta$ gene segments underscore the close evolutionary relationship between these gene families.

References:
number of class I MHC genes in BALB/c. We found that the fourth exon encoding the highly conserved third domain of the class I Dd gene detects 36-40 class I genes, in good agreement with previously published, independent determinations (Fisher et al., 1985; Steinmetz et al., 1982; Winoto et al., 1983). Dot blots and genome blots with copy number controls give results consistent with a J558 family size of 500-1000 VH gene segments. Measurements of the thermal stability of duplexes of the J558 probe with genomic members of the J558 family indicate that the average homology of the members of the J558 family to the J558 sequence itself is 76%. By examining the distribution of bands on genomic blots of germline DNA from the B x D recombinant inbred lines, we have also shown that the VH gene segments we detect with the J558 probe are located on chromosome 12 within one map unit of the other previously mapped VH genes. Furthermore, we note that each band evident on genomic blots of DNA from several mouse strains contains multiple VH gene segments, and that a significant fraction of these bands are polymorphic among the mouse strains tested. These results demonstrate that the germline VH locus of the BALB/c mouse contains at least 1000 VH gene segments. A number of implications concerning both the magnitude and evolution of the VH locus in mouse follow from this demonstration. Since each band evident in the pattern of bands on a genomic blot of mouse germline DNA contains many VH gene segments, irrespective of the restriction enzyme used to generate the pattern, we suggest that the VH gene segments contained in a given band are highly homologous to one another. Since we observe many polymorphic bands containing multiple VH gene segments each among the mouse inbred strains (BALB/c, C57Bl/10, C57L, C3H, A/J, DBA/2) we tested, we also speculate that the gene segments in these bands are very close together or perhaps tandemly linked, and that at least several large-scale duplication and deletion events have occurred during the evolution of the mouse and have been fixed in the various mouse inbred strains.

References:


78. Genes Encoding the VH Region of Anti-α(1+3) Dextran Antibodies

Carol Readhead, Joan L. Klotz, Teresa Geffroy
Roger M. Perlmutter

The antibody response of mice to the polysaccharide α(1+3) dextran is either strong, rapid and dominated by a particular idiotype or slower and weaker with no dominant idiotype. This response is linked to the heavy chain locus, so that mice bearing the allotypic marker Ig-1a are "responders" while those with Ig-1b, c, d, e or f are "non-responders."

It is thought that the non-responders may lack the structural gene that codes for the dominant idiotype, which is defined in terms of the dextran-binding myeloma protein J558 (Schilling et al., 1980). The VH segment sequence of J558 is identical to those of 17 out of 21 dextran-binding myeloma and hybridoma proteins that have been sequenced (Schilling et al., 1980). Thus, the VH gene segments encoding the J558 myeloma protein play a major role in the α(1+3) dextran response.

We have attempted to identify the germline J558 VH gene segment using as a probe the rearranged genomic clone isolated from the J558 myeloma by Roy Riblet. This search has been hampered by the fact that there is a very large number of VH gene segments closely homologous to J558 (see Abstract No. 77). We have constructed a probe consisting of the 5' flanking region of the J558 V gene. Southern blot analysis with EcoRI-cut genomic DNA shows that the 5' flanking region probe hybridizes to two major bands, one at 23 kb and another at 4.8 kb, and that the 4.8 kb band is absent in the non-responder strains of mice. It appears that the VH gene segments in the 4.8 kb band may be involved in the dextran response. Thus, a limited library was made from EcoRI fragments ranging in size from 4-6 kb. A large number of clones were isolated from this library that had VH gene segments homologous to J558 but only a small fraction (10%) of these had 5' flanking sequence homology to J558. These are presently being sequenced. So far, these VH
gene segments are 88-95% homologous to J558 but they are even more closely homologous to each other, some being as close as 99% homologous. These closely homologous \( V_H \) gene segments often occur on the same size EcoRI fragment, indicating that these segments may have been duplicated many times during expansion of the murine dextran \( V_H \) family.

References:

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79. Developmentally Controlled Expression of Heavy and Light Chain Variable Region Genes

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The acquisition of antibody responsiveness to defined antigens follows an orderly developmental sequence in neonatal and young animals. In mice, antibodies specific for certain antigens (dinitrophenyl, azobenzene arsonate, dansyl influenza hemagglutinin) can be induced at birth, while antibodies specific for other antigens do not appear for several days (inulin, phosphorycholine) to weeks \([a(1 \rightarrow 6)\) dextran]. One possible reason for this developmental pattern of antibody expression is an ordered progression of \( V \)-gene rearrangement which would be limited to a few \( V \) genes during early stages of development of the immune response but would gradually increase in time to encompass the entire repertoire of functional \( V \) genes. In order to test this hypothesis, \( V \)-gene expression of a set of pre-B cell and B-cell hybridomas obtained from fetal liver and neonatal liver and spleen was examined.

In initial studies, B-cell hybridomas were evaluated for heavy chain variable region \( (V_H) \) gene expression. RNA from each B-cell line was hybridized to cloned \( V_H \) probes representing five \( V_H \) gene families. These studies revealed that \( V_H \) gene utilization of fetal liver hybridomas was limited to a very few \( V_H \) genes and in fact was dominated by a single \( V_H \) gene family, \( V_H \) 7183. Interestingly, this \( V_H \) gene family is located most proximal to the \( C_v \) locus of all mapped \( V_H \) genes in BALB/c mice. Around the time of birth, the expression of different \( V_H \) gene families by B-cell hybridomas increases dramatically and by day 2 after birth, representatives of each of the five \( V_H \) gene families were detected.

The analysis of \( V_H \) gene expression provided evidence consistent with limited rearrangement of \( V_H \) genes by the earliest B cells that develop in the mouse; however, \( V_H \) gene expression did not appear to be a limiting factor in neonates. An additional component that would determine the specificities of the neonatal antibody response is the diversity of light chain variable region \( (V_L) \) gene expression. To study this question, cloned probes for five \( V_L \) gene families have been prepared. Of the three \( V_L \) gene families that have been tested thus far, one appears to be frequently utilized by fetal and day 1 neonatal B-cell hybridomas but not by day 2 neonatal B-cell hybridomas. The other two \( V_L \) gene families are utilized by a few day 2 neonatal B-cell hybridomas. The majority of \( V_L \) genes transcribed by day 2 neonatal B-cell hybridomas remains undefined. The data on \( V_L \) gene expression thus far support a progression of \( V_L \) gene rearrangement, which parallels that observed for \( V_H \) genes but which may be delayed relative to \( V_H \) gene rearrangement.

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80. Murine Histocompatibility Class I Antigens as a Model System for Cell-Cell Interactions in the Immune System

*Iwona Stroynowski*

Some of the most important steps in the generation of immune responses require specific cell-cell interactions. As an experimental system for these processes, we have chosen to study the mechanisms by which T-cell receptors on cytotoxic T lymphocytes recognize polymorphic transplantation antigens on target cells. Two different types of such interactions are known to occur: 1) allogeneic recognition takes place directly between T-cell receptor and class I molecules, and 2) restriction or dual recognition occurs when the cytotoxic T cell recognizes a foreign antigen (usually pathogenic) simultaneously with the transplantation antigen. It is now well established that T-cell proliferation and activation (or ability to
perform cytotoxic functions) depend on direct contact with the target cells. In recent years, it also became evident that T cells, in turn, also regulate the expression of the molecules with which they can interact. Our research is directed towards quantitative analysis of different factors involved in the cell-cell cooperation.

1. Molecular Dissection of Functional and Polymorphic Regions on Transplantation Antigens

We are interested in identifying the regions on class I molecules that contact T-cell receptor and other functionally important molecules. Our previous studies have shown that the majority of the polymorphic regions recognized by cytotoxic T cells (CTL) are located in the two most external regions (α1 and α2) of the transplantation antigens (Stroynowski et al., 1984). These two regions appear to form a polymorphic functional domain independent of the interactions with the polymorphic regions located on the α3 region. We continued to study the structure-function relationship of individual coding regions of class I mouse major histocompatibility complex proteins by a combination of recombinant DNA, gene transfer techniques, and serologic and functional characterization. To examine the role of α1 and α2 regions in antibody and CTL recognition, the third exon of H-2Dd, Kd, and Ld transplantation antigen genes was replaced by the homologous coding region of the Qa-2-coded class I gene, Q6. We have chosen to carry out the exon shuffling experiments between these two different types of class I genes because they are structurally similar and did not evolve to carry out identical functions. Therefore, it is less likely that the hybrid proteins will fortuitously recreate α1-α2 controlled, functionally important determinants. The replacement of H-2 α2 coding region with its Q6 counterpart had different effects on the expression of the three genes. The mutant H-2Dd gene transfected into L cells was expressed at high levels and retained several of the serologic determinants found on parental H-2Dd and Q6 domains. The serologic epitopes on the mutant H-2Kd-transfected cells were detectable at very low levels, whereas the product of the mutant H-2Ld gene could not be detected at all. Analysis of cells transfected with mutant H-2Dd gene with alloreactive and minor antigen(s)-restricted cytotoxic T cells indicated that the hybrid proteins lost the ability to be recognized by T cells.

Our data (Stroynowski et al., 1985a) suggest that cytotoxic T cells recognize conformational determinants composed of amino acids from α1 and α2 regions. Alternatively, it could be proposed that T cell recognition sites located in a single α1 or α2 region are susceptible to distortion upon α1-α2 interactions. Such susceptibility to conformational changes of the amino-terminal domain of transplantation antigens could be of functional importance for H-2-restricted antigen presentation.

2. Regulation of Class I Antigen Expression

It has been reported previously that various class I molecules (H-2, Qa, and Tla) are under complex regulatory control of interferons, growth factors, glucocorticoids and prostaglandins (reviewed in Wong and Schrader, 1985). Of particular interest are lymphokines produced by activated T cells and secreted into the medium, where they locally modulate expression of MHC antigens. We have begun to study the action of one of these lymphokines, γ-interferon (γ-IFN), on expression of different H-2 molecules. In collaboration with Bette Korber (see Abstract No. 81), we have examined the effects of γ-IFN on cloned hybrid Qa antigens transfected into L cells (hybrid antigens described in Straus et al., 1985 and Stroynowski et al., 1985b). We have demonstrated that γ-IFN induces different Qa hybrid antigens to various degrees and that the ability to be differentially induced is encoded within the 5' half of the Qa genes. We also have shown that the induction of H-2 antigens with γ-IFN may be controlled by sequences located downstream from the promoter as well as the 5' flanking regions.

We plan to extend these analyses to study of other regulatory molecules that may further enhance (α/β interferons) or antagonize (interleukin-3 and glucocorticoids) the action of γ-IFN.

3. Analysis of Tissue-specific Expression and Functions of Non-classical Transplantation Antigen-like Molecules

A majority of class I genes in mouse are encoded by a Tla complex located adjacent to the MHC complex. These genes (TL and Qa) are expressed in a tissue-specific fashion as opposed to transplantation antigens that are found in post-embryonic stages on all cells of the body. The complete tissue distributions and
functions of Tla-encoded antigens are unknown. Some of the Tla complex genes are expressed as secreted products, others as membrane-bound glycoproteins.

We have begun to study tissue specificity of some of these genes by DNA transfection of different cells. In preliminary experiments, we found that one of the Qa genes, which does not express in fibroblasts, is expressed when introduced into mouse hepatoma cell line. The trait responsible for non-expression in fibroblasts maps to the 3' half of the gene. We plan to investigate if TSE (tissue-specific extinguisher) described recently by Killary and Fournier (1984) is involved in the negative regulation of this gene in fibroblasts.

To assess the ability of tissue-specific antigens to perform functions similar to transplantation antigens, we modified the structure of Qa genes allowing them to be expressed nonspecifically as cell-surface antigens. In collaboration with Dr. Carol Readhead, we are introducing the hybrid genes into transgenic mice. We hope that our approach will allow us to get expression of Qa hybrid molecules on all cells of the body and make it possible to study if such molecules can present antigens to T cells and if they function in graft rejection processes.

References:

81. Enhanced Expression of Class I Genes Upon Treatment of Cells with Gamma-Interferon

Bette Korber

The major histocompatibility complex (MHC) of the mouse encodes for a variety of proteins that have important functions in an immune response. The class I genes K, D, and L encode cell-surface glycoproteins expressed on most cells that allow cytotoxic T cells to destroy cells that display foreign antigens. The Qa and Tia genes, also encoded in the MHC, are structurally closely related to K, D, and L; they are not yet defined functionally (Hood et al., 1983).

Gamma-interferon (γ-IFN) is a secreted protein produced by activated T cells, which acts as a lymphokine. It modulates the expression of both class I and class II genes. The transplantation antigens K, D, and L are expressed at higher levels in most cells after exposure to γ-IFN (Wong and Schrader, 1985). It is thought that the IFN-modulated increase in expression is due to both mRNA stability and increased rates of transcription (Friedman et al., 1984).

We are using murine γ-IFN, provided by Genetech, to study the mechanism of induction of class I genes by γ-IFN. In order to study the transcriptional regulation of these genes and to try to ascertain what role the 5'-flanking promoter region has in γ-IFN induction, the 5'-flanking regions of the genes were subcloned to be contiguous to the bacterial gene chloramphenicol acetyl transferase (CAT). These fused class I promoter-CAT constructs were transvected into mouse L cells and stable transformants were generated. The transformants give a twofold increase in the level of CAT activity when the cells have been treated with γ-IFN. Endogenous genes assayed by radioimmunoassay (RIA) are induced three to tenfold, depending on the specific gene, as are intact class I genes introduced into the L cells by transvection of the cosmids that contain them.

A deletion set has been constructed of the 5'-flanking region of the Dd promoter-CAT construct. The purpose of this deletion set is to determine the specific region in the DNA responsible for the twofold induction of the CAT gene. Preliminary studies using transient transvection into NIH3T3 cells indicate an important region, which we are currently studying in greater detail.

A consensus sequence for γ-IFN induction, based on sequence analysis of human genes, has been proposed (Friedman and Stark, 1983). The 5'-flanking sequences of Dd, Ld, and four Qa genes have been determined, and all contain the proposed consensus sequence.

In collaboration with Dr. Iwona Stroynowski, four hybrid Qa/Ld genes constructed so that the 5' end of the gene is contributed by a Qa gene and the 3' end of the gene is contributed by Ld were studied by RIA in transvected L cells to check for response to γ-IFN. The genes were induced at varying levels, depending on
which Qa gene contributed the 5' end. Also, an L^d promoter mutant that is missing the entire 5'-flanking region but is expressed at low levels in transvected L cells was shown to be induced 2.5 fold by γ-IFN when assayed by RIA.

These studies suggest that there are at least two separate components to γ-IFN induction, one found in the 5'-flanking region DNA, as detected by the CAT assay, and one in the coding region, as suggested by the L^d promoter-deficient mutant.

References:

82. Structure of Tla Genes

Douglas A. Fisher

Thymus leukemia (TL) antigens are cell-surface glycoproteins that are expressed only on thymocytes and on some leukemia cells of thymic origin. TL antigens are biochemically similar to transplantation antigens and are encoded at a genetic locus, Tla, that is closely linked to the H-2 complex. The H-2, Qa, and TL antigens are encoded by members of the class I gene family.

Most, if not all, of the BALB/c class I genes have been cloned (Steinmetz et al., 1982). Transformation of the cloned genes into mouse L cells identified two class I genes that encode TL antigenic determinants (Goodenow et al., 1982). We have determined the DNA sequence of both genes (Fisher et al., 1985; D. Fisher, M. Pecht and L. Hood, manuscript in preparation), but find one to be a pseudogene. This surprising result is not yet understood. The other gene, denoted gene 17.3A, encodes a serologically-defined TL antigen. Gene 17.3A is homologous in both sequence and in most intron-exon boundaries with H-2 genes, with the major exception being the cytoplasmic region, where gene 17.3A utilizes one cytoplasmic exon, whereas H-2 genes generally use three. As expected, the DNA sequence of gene 17.3A has diverged considerably from H-2 genes. Two low copy coding region probes from gene 17.3A hybridize to thymus RNA but not to spleen or liver RNA. These Tla-specific probes should prove to be valuable in analyzing RNA species and Tla genes in normal and leukemic cells.

An understanding of the structure of Tla genes will be needed before the function of TL antigens can be determined. We are now able to construct hybrid H-2/TL genes and will attempt to express the TL antigen by, for example, the injection of the Tla gene (or hybrid genes) into mouse embryos. We also will look at the Tla genes from low and high expressor mouse strains. Ultimately, the TL antigen may prove to be an interesting system in which to study gene expression because it is a differentiation antigen expressed in a specific cell type (T cells) in contrast with the ubiquitously expressed H-2 genes.

References:

83. Molecular Studies on the Structure and Function of the Murine Thymus Leukemia Antigen

Stephen W. Hunt III, James Forman

The class I genes can be divided into two main categories: transplantation antigens and hematopoietic differentiation antigens. The transplantation antigens, K, D, and L, are present on most somatic cells and are involved in the T-cell recognition of antigen. Conversely, the differentiation antigens, which include the thymus leukemia (TL) antigen, are found only on subsets of lymphoid cells and their function is unknown. Also, TL antigens are not recognized by alloreactive or H-2-restricted cytotoxic T lymphocytes (CTL). One possible explanation for the inability of TL antigens to serve as targets for CTL is that sequences in the carboxy terminus of the antigen prevent the molecule from functioning in this manner. To test this hypothesis, we constructed a hybrid gene that contains the leader peptide, α1, and α2 exons from the L^d gene and the α3, transmembrane, and cytoplasmic exons from the T13 gene. The hybrid L^d/TL gene and the parental L^d and TL genes were transfected into Ltk^− cells by calcium phosphate precipitation. The hybrid gene encoded a cell-surface product that reacted with monoclonal antibodies specific for the α1 and α2 regions of L^d but was unreactive with a monoclonal
antibody specific for the α3 region. The Ld/TL product did not react with a monoclonal antibody specific for the α1 and α2 regions of TL. Further evidence that the hybrid gene product was encoded by Ld 3′ sequences and TL 3′ sequences was provided by Northern blot analysis. A transcript was identified in RNA isolated from cells transformed with the Ld/TL gene that hybridized with oligonucleotide probes specific for the α2 region of the Ld and the transmembrane region of Tl3. No hybridization was observed with probes specific for the α1 and α2 regions of TL or the transmembrane region of Ld.

We next tested whether the Ld/TL antigen could serve as a target for alloreactive and H-2 restricted CTL. CTL were generated in bulk culture against H-2Ld or against H-2^2d antigens and were tested for their ability to lyse cells transfected with the Ld gene or the hybrid gene. Both anti-H-2Ld and anti-H-2^2d CTL-lysed cells transformed with the Ld and Ld/TL genes but not Ltk− cells. All three targets were lysed by anti-H-2^k CTL, as Ltk− cells are of the H-2^k haplotype. To test for lysis by H-2-restricted CTL, we generated CTL against vesicular stomatitis virus (VSV), which is known to be restricted by H-2Ld. The CTL-lysed, VSV-infected cells transfected with the Ld gene or the hybrid gene but not VSV-infected Ltk− cells. These data indicate that the 3′ portion of the Tl3 gene encodes a functional carboxy terminus for alloreactive and H-2-restricted CTL.

References:


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84. Molecular Basis of the dm1 Mutation in the Major Histocompatibility Complex of the Mouse: A D/L Hybrid Gene

Y. Henry Sun, Robert S. Goodenow†, Minnie McMillan

Mutants of the transplantation antigens have a profound effect on T-cell immune responses. Studies of these mutants have yielded valuable information on the link between biological function and molecular structure. Most mutants carry one to three amino acid substitutions in one of the transplantation antigens. The dm1 mutant under study is unique among all mutants in that both the H-2D and H-2L transplantation antigens are affected. In the mutant, only a single H-2D/L-related transplantation antigen can be identified and show extensive differences from the H-2D and H-2L antigens (Brown et al., 1978; Wilson et al., 1982; Burnside et al., 1984; Hunt et al., 1985).

The mutant dm1 gene was isolated from a cosmid library constructed from dm1 liver DNA. Restriction site mapping and DNA sequence analyses show that the mutant gene is formed by fusion of the 5′ part of the H-2Dd gene and the 3′ part of the H-2Ld gene, with the region in between deleted. The recombination occurred within a region of about 100 bp in the third exon. This region is identical to both the Dd and Ld genes. The recombination could result from either deletion or unequal cross-over by homologous recombination. This gene fusion is analogous to the hemoglobin Lepore variant found in human.

When this cloned hybrid gene is transfected into mouse L cells, serological and biochemical analyses again demonstrated that the antigen expressed is a H-2D/L hybrid molecule and is identical to the mutant molecule identified in the dm1 spleen cells. These results show that the cloned gene is responsible for the mutant phenotype.

Duane W. Sears, Iwona Stroynowski

Secondary mutants of the hybrid dm1 H-2D/L gene, cloned from the B10.D2 (dm1) mutant mouse strain, are being constructed for functional analysis of the T cell recognition sites on H-2 antigens. The dm1 H-2D/L gene is a hybrid of the parental H-2Dd and H-2Ld genes in that the leader exon, the first structural domain exon, and a large part of the second structural domain exon derive from the H-2Dd gene, whereas the remaining 3′ portion of the gene derives from the H-2Ld gene.

By exchanging exons between the mutant dm1 gene and the parental H-2Dd or H-2Ld genes, two secondary mutant genes will be created. One of these genes will encode an H-2 antigen that differs from the parental H-2Ld amino acid sequence at positions 92 (Thr>Ser),
99 (Tyr+Ala), 104 (Gly+Glu) and 114 (Glu+Trp). The other secondary mutant gene will express an antigen that differs from the parental H-2Dd amino acid sequence at positions 155 (Arg+Tyr), 156 (Asp+Tyr) and 169 (Arg+His). The mutant genes will then be introduced into mouse L cells and analyzed with monoclonal antibodies to map serologically-defined determinants. Transfectants also will be infected with reovirus and analyzed in terms of T-cell recognition with allospecific lymphocytes and with virally-restricted T-cell killers. Reovirus, a double-stranded naked RNA virus, elicits potent cytotoxic T-cell response in strains encoding parental molecules (B10.D2-H-2Ld) as well as in mice carrying mutant dm1 antigens (B10.D2-H-2dm1). The cells expressing the two secondary mutant genes will allow us to analyze how the localized amino acid substitutions affect T-cell recognition during the reovirus cytotoxic response.

86. Expression of Class I Genes in Normal Tissues and Transformed Cells
Stephen W. Hunt III, Kurt A. Brorson, Robin Robinson

The study of the class I genes, other than the transplantation antigens, has been hampered by the lack of specific probes. We have chosen to attack the problem of class I gene expression in BALB/c by generation of gene-specific oligonucleotide probes. The transmembrane exon is the most variable exon among the class I genes sequenced to date. It is from this region that we chose to make specific probes. Using an oligonucleotide primer derived from the 3' end of the fourth exon, we sequenced by the chain termination method across the fourth intron (120-150 bp) and through the transmembrane exon (120-140 bp). All but two of the class I genes have been sequenced in this manner. Based on the sequence information, oligonucleotide probes 16 to 21 residues in length were made. Each probe was checked for specificity by hybridization to restriction digests of cloned BALB/c class I genes. The individual probes hybridized to from one to three genes. The hybridization and sequence data indicate that there have been several recent gene duplication or gene conversion events in both the Qa and Tla regions of the BALB/c mouse.

We have begun an analysis of class I gene expression in normal tissues of the BALB/c mouse by probing Northern blots with the oligonucleotide probes. At this time, the results are incomplete and preliminary. We have, however, identified a transcript that hybridized to an H-2 region gene probe. We believe this gene is probably the R gene. We have identified a transcript analogous to the Q10 gene of the H-2Bb haplotype which encodes a liver-specific, secreted class I product (Mellor et al., 1984). In addition, we have observed that both the K and the D genes are induced in murine sarcoma virus-transformed as well as SV40-transformed cells. Further analysis with these oligonucleotide probes should provide us with a picture of the pattern of class I gene expression in both normal tissues and transformed cells.

Reference:

1University of Texas Health Science Center at Dallas.

87. Identification of Sequences Important for the Sorting, Transport, and Cell-Surface Expression of H-2 Molecules
Martha C. Zuniga

The histocompatibility antigens are integral membrane proteins on the surfaces of almost all mammalian somatic cells. Many of the details of the biosynthesis of H-2 proteins have been elucidated, such as their association with β2-microglobulin (β2-M) on the rough endoplasmic reticulum (ER), and the glycosylation events that occur during the maturation of the functional cell-surface forms. H-2 proteins share many structural features in common with other integral membrane proteins, including a signal sequence and a hydrophobic transmembrane (TM) region, which is flanked on either side by charged residues. The availability of cloned genes, specific monoclonal antibodies, and functional assays for H-2 proteins makes them ideal for studies on the biogenesis of integral membrane proteins. We have used in vitro mutagenesis to assess the contributions of a cluster of four basic amino acids on the cytoplasmic side of the TM region to the sorting, transport, and stable cell-surface expression of H-2Ld. The truncated variants constructed for these studies encode H-2Ld proteins lacking most of the 31 amino acid cytoplasmic tail and having zero, two (serine-glutamic acid), or four (lysine-arginine-serine-glutamic acid) residues on the...
C-terminal side of the TM segment. Stable mouse L cell lines expressing these genes have been generated. Detailed analyses of these clones show that the cluster of basic residues on the cytoplasmic side of the TM segment enhances high levels of cell-surface expression of H-2L\(^d\) but is not required for it. An H-2L\(^d\) molecule lacking the entire cytoplasmic tail can be expressed on the cell surface, where it has a turnover rate that is indistinguishable from that of H-2L\(^d\) molecules with cytoplasmic tails. Pulse-chase and Endo H studies show that the lower cell-surface expression of truncated H-2L\(^d\) molecules correlates with a slower rate of transport through the Golgi apparatus. Our results suggest that the charged residues in the cytoplasmic side of the TM region constitute part of a sorting sequence that facilitates the transport of H-2L\(^d\) to the cell surface.

88. Analysis of Intracellular Transport of Integral Membrane Proteins in Cloned Mouse L Cells

Martha C. Zuniga

We have recently generated cloned cell lines derived from mouse L cells transfected with a novel H-2 protein, which has an intact membrane-spanning (TM) region but no cytoplasmic tail. Two thirds (14/21) of these cell lines fail to express the tail-less H-2 molecule on the cell surface but express it intracellularly where it undergoes normal glycosylation and has a long half-life. The remaining seven clones express the tail-less H-2L\(^d\) on the cell surface. Recent cDNA and genomic sequence data show that Thy-1 antigen is an integral membrane protein with no cytoplasmic tail. Since Thy-1 is limited in its tissue distribution, it is interesting to consider the possibility that the sorting pathway by which tail-less integral membrane proteins are transported to the cell surface is utilized in some cell types but not others. The fibroblast clones that express tail-less H-2L\(^d\) on the cell surface may have deviated in their sorting mechanisms during the long-term culture of the parent line. We are testing these stable L-cell clones, which do and do not express the tail-less H-2L\(^d\) on the cell surface, for the cell-surface expression of Thy-1. Since Thy-1 driven off its own promoter is expressed well in lymphoid and neuronal cell lines but is poorly expressed in L cell (Evans et al., 1984), we are preparing a Thy-1 construct whose endogenous promoter is replaced by the promoter of the mouse metallothionein (MT) gene. Transformed cells will be analyzed by cell-surface radioimmunoassay and immunoprecipitation with Thy-1-specific monoclonal antibodies and by biosynthetic studies. Further characterization of these clones will determine their usefulness for further analysis of the intracellular transport of integral membrane proteins in mammalian cells.

Reference:

89. Study on the Structure-Function Relationship of Class II MHC Molecules

Bernard T. M. Vernooy, Bernard Malissen, Nilabh Shastri

Class II MHC molecules are a group of cell-surface molecules that are involved in the generation of an immune response against a vast array of foreign antigens. Together with the antigen, the class II molecule stimulates T cells that facilitate the immune response. This stimulation is specific: a T cell can only be stimulated by one particular combination of class II plus antigen.

The class II molecules consist of two chains, \(\alpha\) and \(\beta\), that each contain two external regions, \(\alpha_1\) and \(\alpha_2\) and \(\beta_1\) and \(\beta_2\). Sequence comparisons have shown that the different \(\alpha\) chains are fairly homologous, as are the \(\beta\) chains. Most of the amino acid differences that are present are located in the first regions, \(\alpha_1\) and \(\beta_1\) (Benoist et al., 1983; Gustafsson et al., 1984). In our study of the structure-function relationship of class II molecules, we focused on the \(\beta_1\) region.

The exons encoding the \(\beta_1\) regions of \(\alpha^k\) and \(\alpha^d\) have been cloned into appropriate vectors that will allow recombination (in E. coli) to occur between the \(\beta_1\) sequences. Following the recombination, the \(\alpha^k_\beta^d\) gene sequences will be characterized by nucleotide sequencing. Next, these hybrid exons will be individually placed into \(\alpha^k\) genes that lack the normal \(\beta_1\) exon. The hybrid \(\alpha^k_\beta^d\) gene and the \(\alpha^k\) gene can then be transfected into mouse L fibroblasts. \(\alpha^k\)-transfected L cells are capable of expressing the I-\(\alpha^k\) molecule on the cell surface and of presenting certain antigens to T cells that are I-\(\alpha^k\)-restricted (Malissen et al., 1984).

By using L cells transfected with the hybrid genes
and I-\(A^k\)-restricted T-cell clones, we will be able to map those parts of the \(\beta\)1 region involved in the activation of the specific T cells.

References:

90. Analysis of I Region Recombinants of the Murine MHC: The I-J Structural Gene Is Not Encoded Between the I-A and I-E Subregions
Joan A. Kobori, Erich C. Strauss, Karyl Minard

Restriction enzyme site polymorphisms have been used to correlate the molecular maps of the I region of the murine major histocompatibility complex (MHC) with the genetic map derived from analyses of recombinant mouse strains (Kobori et al., 1984). These data indicate that the I-J subregion is limited to 1.37 kb and is contained entirely within the class II gene, \(E^B\) (Biology 1984, Abstract No. 64). We have sequenced the I-J subregion from partial \(E^B\) clones derived from the two mouse strains initially used to define the I-J subregion: B10.\(A(3R)\), I-\(J^B\), and B10.\(A(5R)\), I-\(J^K\). These regions are identical; hence, the distinct I-\(J^B\) and I-\(J^K\) molecules cannot be encoded by the immunogenetically-defined I-J chromosomal region of the MHC. DNA sequence analysis permits us to define the I-J subregion as a 1.0 kb stretch of DNA. Analysis of the DNA sequences of three parental (C57BL/10, B10.A and B10.D2) and four I region recombinants (B10.\(A(3R)\), B10.\(A(5R)\), B10.GD and B10.\(A(6R)\)) indicates that three of the recombinant strains recombined within a 1 kb region of DNA, supporting the notion that a hotspot for recombination exists in the I region.

Reference:

Chia-lam Kuo

Our interest in studying the functional expression of T-cell receptor genes requires developing an efficient system by which we can stably introduce exogenous DNA into T cells at high frequency for expression. Since histocompatibility H-2 antigens are cell-surface glycoproteins and specific monoclonal antibodies are available, this allows us to evaluate the transfection protocols by measuring the level of expression of introduced H-2 genes on the transformed cell surface.

I have transferred the cloned mouse \(A^k_a\) and \(A^k_b\) genes (Malissen et al., 1984), which encode the class II I-\(A^k\) molecules, into mouse EL4, a thymoma of helper lineage, by the electroporation method (Potter et al., 1984). Monoclonal alloantibodies defining distinct polymorphic regions of the I-\(A^k\) molecules (Pierres et al., 1980) were used to show that the I-\(A^k\) molecules are expressed on the cell surface. I have also obtained the expression of a cloned class I gene, the L\(d\) gene, on the surface of mouse thymoma. Whether the I-\(A^k\) molecules expressed on mouse T-cell transformants are biochemically indistinguishable from their normal counterparts, and whether the T-cell transformants expressing I-\(A^k\) molecules can present certain antigens to I-\(A^k\) restricted T-cell hybridomas will be further investigated. In addition, a question of interest is why T cells which normally do not express class II genes can express exogenous class II DNA after DNA-mediated transfection.

I have also been cloning T-cell receptor genes from influenza virus-specific cytotoxic and helper T-cell clones. Upon isolation of these clones, the functional assays for the receptor on the T cells transformed with \textit{in vitro} mutagenized T-cell receptor genes by the DNA-mediated gene transfer technique mentioned above will then be performed to determine the amino acid residues involved in antigen binding and MHC recognition.

References:
92. Influence of la Polymorphism on the Specificity of T Cells Induced by Synthetic Peptides of Lysozyme

Nilabh Shastri, Deborah Munt, William D. Nelson, Suzanna J. Horvath

Studies on the specificity repertoire of T-helper cells have repeatedly demonstrated a major influence of the polymorphic la molecules encoded by the major histocompatibility complex (MHC). Thus, pairs of MHC congenic mouse strains show dramatic differences in responsiveness to the same antigen, a phenomenon referred to as an immune response (Ir) gene defect. Conclusive distinctions have not been possible between the two major hypotheses accounting for these differences. Response status could be a reflection of either the available T-cell repertoire or the ability of antigen-presenting cells to stimulate specific T cells. Both the intrathymic development of the T-cell repertoire and the antigen-specific activation of T cells by antigen-presenting cells are known to be intimately linked to the la molecules encoded within the MHC.

We chose the C57BL/6 (B6) and B6-C-H-2bm-12 (bm-12) mouse strains to study T cell responsiveness to a synthetic peptide T11 (a.a. 74-96) of hen lysozyme. Since these strains differ in only three amino acid substitutions in the B subunit of the I-A molecule, it would be possible to precisely determine the polymorphic differences in the la molecule responsible for the observed differences. The choice of the antigen was dictated by the availability of a large number of synthetic variants and earlier information on the specificity profile of B6 T cells (Shastri et al., 1984, 1985).

Our studies have identified differences in responsiveness to synthetic variants of T11 peptide and in the antigenic specificity of T-cell clones derived from both B6 and bm-12 mice. All T clones were exquisitely specific only for the parental la molecule and failed to recognize the antigen presented by antigen-presenting cells from the congenic partner, demonstrating the dramatic effect of la polymorphism. Future studies are directed towards analysis of receptor genes of the T cells and the la molecules to define the structural parameters responsible for these reactivity differences.

References:

93. Antigen Presentation Function of la-Expressing Mouse Fibroblasts

Nilabh Shastri, Bernard Malissen, Deborah Munt

It is believed that the T-cell receptor co-recognizes regions of the antigen and the MHC-encoded la molecules. This phenomenon is referred to as MHC-restricted recognition of antigen. The la molecules, composed of the a and B subunits, are known to be extremely polymorphic within the species and are known to influence the specificity of the induced T-cell responses. The molecular mechanisms underlying this phenomenon are poorly understood.

Earlier studies (Malissen et al., 1984) have shown that mouse fibroblasts, which do not express the endogenous la genes, do express la molecules on the cell surface following gene transfer. Moreover, these la-expressing fibroblasts can serve as antigen-presenting cells in stimulating antigen-specific, MHC-restricted T-cell responses. These observations have opened up a new approach to the study of 1) the structural features of the la molecule relevant to T-cell activation by using in vitro modified genes, and 2) the biochemical events associated with la-restricted antigen recognition.

Mouse fibroblasts were transfected with the E_k and E_d genes consisting of individual exons derived from either k or d haplotypes. Using T cells restricted by either the E_k or E_d molecules, it was shown that substitutions only in the first extracellular region (a1 exon) of the B subunit accounted for the allelic differences in the ability to stimulate T cells. This agrees well with the fact that 21 of the 22 amino acid substitutions between the B subunits of the k and d haplotype-derived molecules reside in the first region. Future studies are directed toward determination of the precise substitutions responsible for the allelic differences as relevant to T-cell activation utilizing site-specific mutagenesis techniques.

In another study (Shastri et al., 1985) comparing antigen presentation by I-A_k-transfected fibroblasts with physiological antigen-presenting cells, it was observed that while both cell types were competent in
the presentation of a lysozyme peptide to T clones, the fibroblasts in contrast to spleen cells failed to present native lysozyme. The same transfectant cells have been shown to present other native proteins, suggesting that proteins differ in their stringency of processing requirements. Further studies are under way to determine both the structural features of the antigen and the lesion in the transfected fibroblasts responsible for these differences.

References:

94. Integration and Expression of Microinjected Myelin Basic Protein Gene in the Shiverer Mutant Mouse
Naoki Takahashi, Carol Readhead

The shiverer mutation is one of several that disrupt myelination and consequently neurologic function in the mouse. Shiverer homozygotes appear unaffected until about 14 days after birth, the time of onset of major myelination of the central nervous system. Then the phenotype consists of neurologic dysfunction of increasing severity, beginning with an occasional shiver when the animal is active or disturbed, and progressing to tonic seizures and premature death. The most striking biochemical defect in shiverer mice is the absence of myelin basic protein (MBP), which is one of the major protein species in myelin.

Previous reports demonstrate that the brains of shiverer mice contain greatly reduced amounts of MBP mRNA, and that five out of six exons of MBP gene are deleted in shiverer genome (Roach et al., 1983, 1985). The wild-type mouse MBP gene has been cloned and sequenced (Takahashi et al., 1985). We have injected this gene into the pronucleus of shiverer embryos and transferred these to pseudopregnant foster mothers (Constantini and Lacy, 1982). Several shiverer mice have integrated the MBP gene into their genome. The transgenic mice are presently being analyzed.

References:

95. Cloning the Human Myelin Basic Protein Gene
Brian Popko, Naoki Takahashi

Myelin is a multi-layered membrane structure that surrounds the axons of most neurons of the central and peripheral nervous systems. Its major function is to facilitate the rapid conduction velocities of nerve fibers. Thirty percent of the protein in isolated central nervous system myelin is myelin basic protein (MBP). MBP is believed to play an integral role in the maintenance of myelin structure. MBP is found in mice in four forms of 14.0, 17.0, 18.5, and 21.5 kd. The four forms of mouse MBP are believed to be encoded by a single gene which spans approximately 35 kb of DNA (Takahashi et al., 1985). The four forms of mouse MBP apparently arise through differential splicing of a single species of MBP transcripts. In humans, only the 18.5 kd species of MBP has been identified.

We have set out to clone the human genomic MBP gene. These studies were initiated to determine whether the human MBP gene lacks the exon that encodes the 21.5 kd MBP protein or whether the differential species distribution of MBP proteins reflects species-specific RNA-splicing preferences.

A human cosmids library has been constructed from HeLa cell DNA and when screened with a cDNA clone of the rat MBP gene, a single positive clone was identified. This cosmid contained the first exon of the MBP gene plus approximately 20 kb of upstream sequences. Two additional human cosmids libraries have been screened, and five more positive clones identified. These five cosmids also contained only the first exon of the human MBP gene. In an effort to bypass the apparent inability to clone the 3' region of the human MBP gene in cosmid vectors, we have recently constructed a λ library from HeLa cell DNA, and we are in the process of screening for the remainder of the human MBP gene.

References:

96. Purification and Characterization of the Membrane Receptor Protein for Platelet-derived Growth Factor
David D. Woo
Platelet-derived growth factor (PDGF), the cellular
homologue of p28\textsuperscript{sis}, is the major serum mitogen required for the \textit{in vitro} growth of mesenchymally-derived fibroblasts and arterial smooth muscle cells. The biological and oncogenic activity of PDGF is mediated via interaction with specific cell-surface receptors. The PDGF receptor is a 185,000 dalton integral membrane glycoprotein. PDGF also stimulates the phosphorylation of a 185,000 dalton protein in cell membranes.

We initiated studies on PDGF receptor using the mouse fibroblast cell line, NR6, which possesses 2.5-3.0 x 10\textsuperscript{5} PDGF receptors per cell but no detectable receptor for epidermal growth factor.

Purification of the receptor was accomplished by affinity purification of galactosyl-glycoproteins from a Triton X-100 extract of 80 gm of NR6 cells using a ricin Fractogel column. This procedure yields ~100 micrograms of PDGF receptor at ~10% purity. Gel permeation HPLC chromatography on TSK 4000 columns in 0.05% Triton X-100, 100 mM sodium phosphate buffer, pH 7.0, was then used to remove contaminating proteins. This yielded highly purified PDGF receptor, as judged by silver staining after SDS-PAGE. The $^{125}$I-PDF binding activity coeluted with PDGF-induced tyrosine protein kinase activity in the size-based HPLC separation.

Hydrogen fluoride deglycosylation of the purified PDGF receptor indicated that the polypeptide moiety is about 96,000 daltons in molecular mass. Isoelectric focusing studies showed that the intact receptor has a pI = 5.6 before deglycosylation. Two-dimensional phosphopeptide analysis of the purified PDGF receptor, autophosphorylated in the absence and presence of PDGF, revealed that two distinct tyrosine-containing peptides were phosphorylated in response to added PDGF. No additional serine or threonine phosphorylation was detected. Parameters influencing PDGF binding to and the protein kinase activity of the purified PDGF receptor are currently being defined.

97. cDNA Cloning of Platelet-derived Growth Factor Receptor

David D. Woo, Paul Tempst, Lance Fors, Hilde Cheroutre, David B. Teplow, Ruedi Aebersold, Stephen B. H. Kent

As part of our investigation into the role of platelet-derived growth factor (PDGF) and its receptor in atherosclerosis (Ross and Glomset, 1976), wound healing (Ross and Glomset, 1976), and cancer biology (Waterfield, 1983), we have undertaken to characterize the primary structure of PDGF receptor by applying a combination of protein chemical and molecular biology techniques.

PDGF receptor protein was purified to homogeneity from the NR6 cell line. The purified receptor was digested with trypsin and the tryptic peptides then separated by reverse phase HPLC. Selected peptides were then sequenced by automated Edman degradation on a gas phase protein sequenator followed by HPLC analysis of the resultant PTH-amino acids. Reverse translation of these amino acid sequences according to human codon usage frequencies guided the automated chemical synthesis of complementary oligonucleotide probes.

Six different probes, ranging from 27 to 63 nucleotides in length and with varying degeneracies, were used to screen an oligo(dT)-primed $\lambda$gt10 cDNA library made from NR6. Two clones, with 1.8 kb and 0.9 kb inserts, were found to hybridize consistently with their respective probes after repeated rounds of re-screening. They were subcloned into the EcoRI site of M13mpl8 and pUC8 for dideoxy sequencing and plasmid production, respectively. To rapidly determine the authenticity of these clones, the purified inserts from pUC8 clones were digested with Sau 3A and subcloned into the BamHI site of M13. Positive clones were identified by hybridization with the respective oligonucleotide probe and are currently being sequenced. pUC8 clones will be nick translated for use in Northern analysis to obtain information on the size, distribution, and abundance of the PDGF receptor gene transcript in both normal and malignant tissues.

References:


98. A Sib Selection Procedure to Isolate Genes Using Antibodies

Ulf Landegren

Isolation of genes coding for proteins that are only defined by antibodies is a recurring task. Several approaches can be taken, including purification of the protein and determination of its amino acid sequence.
From this, oligonucleotide probes can be synthesized that will permit identification of cDNA clones. Alternatively, stable transfectants, ectopically expressing the gene product, can be developed and used to isolate the cDNA. Finally, bacterial expression vectors may be employed to develop gene banks that can be screened with antibodies.

I am currently investigating the feasibility of using a sib selection procedure to isolate clones encoding a given gene product from a cosmid library. The principle employed is to prepare DNA from pools of cosmids and to use them to transfect fibroblast L cells. The transient expression of genes thus introduced can subsequently be monitored by staining for a given antigen. The positives are visualized using an enzyme-linked reaction. A positive pool can then be subdivided for a second round of selection. Since each round of selection requires only a few days and great numbers of samples can be handled simultaneously, this sorting would be a fairly rapid procedure. I have shown that the immunological detection employed allows a clear-cut distinction of cells expressing the gene product sought. This makes it possible to screen a very large number of L cells for a rare transfectant.

Using the gene for a class I mouse transplantation antigen added at a mass ratio of \(1/3000\), about 1000 positives are observed among \(3 \times 10^5\) plated L cells. This suggests that genes occurring at a far lower ratio should be detectable.

The transient expression assay should enhance the frequency of positives as compared to that observed in stable transfection. In addition, it is likely that it will permit expression of some gene products normally not present in fibroblasts. Using a modification of the procedure, it might be possible to detect also molecules that have not assembled on the cell surface, but are present as cytoplasmic proteins. Receptors, defined by their ability to bind a given ligand, could be visualized similarly.

99. A Method for Rapid Restriction Site Mapping

Yi Henry Sun

Restriction site mapping is one of the most fundamental and important ways of characterizing a cloned DNA fragment. A number of strategies have been employed. I have developed a restriction site mapping method that is rapid, circumvents the difficulties of existing methods, and has general applicability. The method is a modification of the end-labeling and partial-digestion methods of Smith and Birnstiel (1976) and the indirect end-labeling method of Rackwitz et al. (1984). The method was originally developed to determine restriction sites in large DNA fragments cloned in the cosmid vector pTL5, but the principle can be easily applied to DNA fragments cloned in other vectors.

Basically, the cosmid DNA is first linearized by a restriction enzyme that cleaves once in the vector but not in the insert. Either of the two unique restriction sites in pTL5, SalI and ClaI, can be used. The linearized cosmid DNA is partially digested with the restriction enzymes to be used for mapping, electrophoresed in an agarose gel, blotted onto nitrocellulose paper according to Southern (1975), and hybridized separately to two radioactively-labeled, end-specific oligonucleotides. The two oligonucleotides are each 40 bases in length and are complementary to sequence flanking either side of the SalI (or ClaI) recognition site. Each oligonucleotide will hybridize to a set of fragments, all with a common terminus but of different lengths, forming a ladder of bands which can be detected by autoradiography. The positions of all the restriction sites relative to the SalI (or ClaI) site can easily be deduced from this partial digest.

This method avoids the laborious steps required by the method of Smith and Birnstiel (1976). Also, unlike the method of Rackwitz et al. (1984), it is not limited to only \(\lambda\) and cosmid clones. The SalI and ClaI sites chosen in pTL5 are on a fragment containing the tetracycline-resistance gene derived from the plasmid vector pBR322. So the two sets of oligonucleotides can be used for any pBR322-derived, tetC vectors. Other rare-occurring restriction sites can be chosen for other vectors, and a limited number of oligonucleotides corresponding to these will cover most of the commonly used cloning vectors.

References:
100. Mapping of Genes by Pulse Field Gradient Gel Electrophoresis

Eric H. C. Lai, Lloyd M. Smith

The variable regions of immunoglobulins and T-cell receptors are encoded by distinct germline gene segments—variable (V), diversity (D) and joining (J). These genes are scattered over a large region along the chromosomes and an understanding of the functions and the mechanisms responsible for the rearrangement of these genes requires a knowledge of the physical distances separating them. The diversity and joining segments of immunoglobulins and T-cell receptor \( \delta \) chains have been mapped. However, the precise location of the variable segments relative to the diversity segments is currently unknown. Previous attempts at gene mapping by "chromosome walking" and genetic linkage have proven to be tedious and frustrating.

Recently, a new type of agarose gel apparatus that employs two alternately pulsed, inhomogeneous, orthogonal electric fields has been developed (Carle and Olson, 1984; Schwartz and Cantor, 1984). This apparatus allows the separation of DNA molecules of sizes from 30 to 3000 kb and has been used to separate yeast and trypanosome chromosomal DNA molecules. In our laboratory, we have constructed and modified the geometry of the electrodes to improve the resolution of the large size DNA molecules. Southern blot analysis is being used in conjunction with this gel apparatus to determine linkages in the gene systems described above.

References:

101. Specific-Primer-directed DNA Sequencing

Erich C. Strauss, Joan A. Kobori, Gerald Sliu, Suzanna Horvath, Marilyn Tomich, Carol Graham

A simple procedure for DNA sequence analysis based on the Sanger chain-termination method was developed and analyzed. This procedure utilizes inserts of 3-4 kb of DNA cloned into M13 bacteriophage vectors. After the sequence of the first 600-650 bp of the insert DNA has been determined with the commercially-available universal vector primer, a specific oligonucleotide is synthesized utilizing the sequence data obtained from the 3' end of the sequence and used as a primer to extend the sequence analysis for another 600-650 nucleotides. Additional primers are synthesized in a similar manner until the nucleotide sequence of the entire insert DNA has been determined. Confirming sequence on the second strand can be obtained very rapidly using appropriate complementary oligonucleotides spaced every 600-650 base pairs. General guidelines for the selection of oligonucleotide length and composition and the use of unpurified primers were studied. Oligonucleotides varying from 13-88% GC content primed DNA synthesis equally well. The optimal length of the oligonucleotide primer was 15-20 nucleotides. The synthetic stepwise yield of a 15-20 mer is approximately 98% and thus the crude product can often be used without gel purification. The use of highly purified single-stranded template DNA along with the specific-primer-directed approach to dideoxy-nucleotide sequence analysis reduces considerably the time required for the analysis of large segments of DNA.

102. Application of Microchemical Technology to Proteins of Biological Interest


The goal of our work on microchemical instrumentation and microchemical methods has been to provide the technical means to study proteins of great biological interest present in extremely small amounts. We have focused on methods of protein isolation and sequence analysis for the purposes of understanding structure/function relationships within single proteins, of elucidating structural relationships between proteins, and for use in conjunction with molecular biological analyses of the structural genes coding for the proteins being studied.

Sequencing polypeptides available at only microgram or nanogram levels requires improvement of methods of sample preparation, peptide production, sequenator design, Edman chemistry, PTH analysis, and data acquisition/analysis. Our substantial progress in these areas is discussed within the specific abstracts that follow. Here we report examples of the successful application of these improved methodologies to a variety of previously problematic proteins (see
Table 1. The samples presented include hydrophobic integral membrane proteins (liver gap junction, PDGF receptor) and their peptides, hydrophilic peptides (myelin basic protein), and an extremely scarce polypeptide, nerve differentiation factor. The samples have been prepared using two variations of electrophoretic blotting onto chemically-activated glass fiber sheets (electroblotting), by HPLC followed by spotting onto polybrene-coated discs, and by spotting onto quaternary ammonium (QA) derivitized glass fiber discs. All samples were sequenced at the 1-10 picomole level and provided information of use in the elucidation of primary structure and homology relationships, and/or in the design of probes for cloning of, or confirmation of the cloning of, the respective structural genes.

<table>
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<th>Method of Preparation</th>
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103. Protein Microsequencing Methodology: Isolation of Proteins

Ruedi Aebersold, David B. Teplow, John Kim, Wade Hines, Stephen B. H. Kent

Currently, proteins can be sequenced at levels as low as 5-20 picomoles of sequenceable material using the gas phase sequenator (Hewick et al., 1981). Isolation of such small amounts of pure protein in a form suitable for sequencing presents a considerable technical challenge.

We have developed a new approach to the isolation of proteins for microsequencing. The method consists of electrophoretic transfer ("electroblotting") of proteins or their cleavage fragments onto chemically modified glass filter paper sheets immediately after their separation by gel electrophoresis. The proteins are immobilized on the glass fiber sheets by ionic or covalent interactions.

Two sets of conditions have been worked out. The first method, high pH blotting, is used to isolate proteins separated by SDS-PAGE or two-dimensional gel electrophoresis. The second method, low pH blotting, is used to isolate proteins after isoelectric focusing in immobilized pH gradients.

A wide range of proteins can be prepared in this fashion with no apparent restriction due to solubility, size, charge, or other intrinsic properties of the proteins. As little as 50 ng of the transferred proteins can be detected by suitable staining procedures. After detection, the protein-containing bands or spots are cut out and inserted directly into the gas-liquid-solid phase sequenator. The piece of glass fiber sheet acts as a support for the protein during the sequencing. Extended sequencing runs can be obtained from such samples with improved stepwise yields and lower backgrounds, and the staining procedures do not
interfere with sequence determination analysis of the resulting derivatives. The method has also been used to determine the sequence of fragments from proteins with blocked amino termini.

Reference:

104. Approaches to Subpicomole Protein Sequencing


Many biologically important cell-surface receptor proteins occur in very low abundance. Those currently amenable to study are expressed in amounts of about 100,000 molecules per cell in carefully selected high expressor cell lines. Many other receptors of great biological significance occur at only 10-1000 molecules per cell. Current sequencing levels (5-20 picomoles) must be lowered about 1000 fold in order to give information about these rare proteins. To achieve this 10-100 femtomole sensitivity, all aspects of protein sequencing methodology must be improved. With this goal in mind, we have undertaken a systematic program of research in the following areas: 1) separation of proteins by SDS-PAGE, immobiline IEF, or two-dimensional gels, followed by electroblotting onto activated glass fiber sheets for high yield isolation of subpicomole amounts of proteins in covalently-immobilized form directly suitable for sequencing; 2) chemical deglycosylation techniques for picomole amounts of proteins compatible with these new isolation techniques; 3) design and construction of improved protein microsequenators; 4) use of fluorescent and cryptic-functionality (L’Italien and Kent, 1984) Edman reagents; 5) modified reaction conditions for accelerated Edman degradation; 6) alternative procedures for the analysis of the resulting amino acid derivatives; 7) enhanced visible and fluorescence detection of these derivatives; and 8) digital acquisition and manipulation (identification and quantitation) of the analytical data sets, and deduction of the amino acid sequences.

Reference:

105. Protein Microsequencing Methodology: Improved Edman Chemistry

Ruedi Aebersold, Lloyd M. Smith, Karen Parker, Vincent R. Farnsworth, Stephen B. H. Kent

We are continuing our efforts to improve the overall sensitivity of protein sequencing. One limiting factor of current protein sequencing technology is the sensitivity of detection of PTH-amino acids, the degradation products of the Edman chemistry. Attempts to increase the speed and sensitivity of protein sequencing are critically dependent on the nature of the Edman reagent used. An ideal compound should have the following characteristics: i) reactivity comparable with PITC; ii) the reagent and the resulting derivatives should be stable and soluble in solvents used in sequencing; and iii) contain a fluorophoric portion to yield highly detectable derivatives. In order to systematically develop such a reagent, we have modified and programmed a protein sequenator with a highly versatile controller unit so that a variety of coupling and/or cleavage conditions can be evaluated. We have synthesized dipeptides, as well as extended model peptides, on glass and polystyrene solid supports as tools to systematically investigate coupling and cleavage rates of prospective reagents in the sequenator as well as in manual sequencing experiments. We have also worked out several chemical modifications of glass fiber papers in order to covalently immobilize proteins and peptides. These modified glass fiber papers will then act as protein supports in the gas phase sequenator cartridge. Among the functionalities that have been introduced to the solid support are 1,4-phenylene-diso-thiocyanate, aliphatic and aromatic primary amino groups and diazophenyl groups.

106. Structural Characterization of Corticotropin-releasing Factor (CRF) and Related Peptides from Human Placenta

Paul Tempst, Dorothy Krieger, Tony Liotta, Stephen B. H. Kent

Material possessing potent CRF-like activity has been prepared from human placental tissue by a multi-step HPLC isolation procedure. Three distinct fractions were prepared. The first contained material indistinguishable from rat CRF. Extensive fragmentation, amino acid analysis, and protein sequencing determined 36 of the presumed 41 amino acid residues;
these were identical to the sequence deduced from a human genomic DNA clone (Shibahara et al., 1983). Bioassay data showed that this peptide, which is normally released from the hypothalamus and plays a critical role in the endocrine response to stress, is released from the placenta into the circulation in the third trimester of pregnancy. The other two fractions from the original isolations contained peptides about the same size as CRF and of uncertain biological activity. N-terminal sequence determination and computer searching of a sequence data bank showed them to be cytochrome oxidase polypeptide VIIIa (47 residues) and a C-terminal fragment of human γ-hemoglobin chain (47 residues). These may be artifacts of the preparation procedures. However, the first of these showed significant homology to CRF (8 out of 15 residues) and the possibility that these molecules play biologically significant roles in a fashion similar to exorphins is being investigated.

Reference:

1Division of Endocrinology, Mt. Sinai Medical School, New York.
2Deceased.

107. Multidimensional Microscale HPLC Purification and Sequence Analysis of Platelet-derived Growth Factor Receptor and Its Tryptic Peptides

Paul Tempst, David D. Woo, David B. Teplov, Ruedi Aebersold, Stephen B. H. Kent

The growth-promoting and postulated oncogenic activities of platelet-derived growth factor (PDGF) are mediated through interaction with a cell surface PDGF receptor (PDGF-R). This receptor is a 185 kDa intrinsic membrane glycoprotein with PDGF-dependent tyrosine kinase activity. To understand the function of this receptor and to assist in the cloning of its gene(s), we have initiated studies of its primary structure. The PDGF-R was purified from Triton X-100 solubilized mouse fibroblasts by medium performance affinity chromatography on a TSK fractogel-ricin B column, followed by size exclusion chromatography on a TSK 4000 SW column in the presence of SDS. Parallel analytical size exclusion runs were done in the presence of Triton X-100 to monitor the biological activity. Because of the extreme insolubility of the purified receptor, methods to produce fragments were developed that did not involve precipitating or drying the protein. Thus, the receptor was alkylated in 3 M urea, dialyzed against decreasing concentrations of urea, cleaved with trypsin in 2 M urea and immediately injected on a Vydac C4 column. Peptides were further purified by rechromatography on Vydac C4, Vydac C18 and Vydac diphenyl columns. Starting with 50-60 micrograms (250-300 pmoles) of purified receptor, 25 peptides were isolated and sequenced, yielding over 240 residues of sequence.


David B. Teplov, Ruedi Aebersold, Paul Tempst, Stanley B. Prusiner, Stephen B. H. Kent

Scrapie is a progressive, degenerative neurological disease of sheep. The etiologic agent is thought to be a novel proteinaceous pathogen termed a prion, which may also be involved in causation of Creutzfeld-Jakob disease. PrP 27-30 is a polypeptide of apparent molecular weight 27,000-30,000, which is the major protein species associated with infectious prion preparations. To study the relationship of PrP 27-30 to prions and its role in scrapie infection, we have examined the primary structure of the protein and peptide fragments thereof (Prusiner et al., 1984). Based on the sequences obtained, we have synthesized peptides and used these to produce specific antisera and monoclonal antibodies. We have also synthesized oligodeoxynucleotide probes which have been used to clone the structural gene for this polypeptide (Oesch et al., 1985).

The gene cloning work has demonstrated the presence of a cellular gene and a corresponding mRNA in brains of both infected and uninfected hamsters. This has a profound impact on our view of slow virus infections in general, and scrapie in particular. It is now clear that two non-identical homologues of PrP 27-30 exist in brains of normal and infected animals. We hope to elucidate the structural differences between these homologues and by this approach gain an understanding of the mechanism by which scrapie and other slow virus diseases are triggered.

References:

1Department of Neurology, University of California, San Francisco, CA.
109. Structure and Function of a New Insulin-like Growth Factor Peptide

David B. Teplow, Vince W. Hylka1, Daniel S. Straus1, Karen Parker, Stephen B. H. Kent

The insulin-like growth factors (IGF) are members of a family of proteins that include insulin, IGF-I, IGF-II, and relaxin. Supernates from cultured rat liver cells (BRL-3A) contain IGF activity as measured by their capacity to induce tritiated thymidine uptake in cultured hamster cells (N1L-8). This activity elutes in four distinct peaks when BRL-3A supernates are subjected to ion exchange and size exclusion chromatography. We have sequenced the peptides from two of these peaks and determined that each peak contains different ratios of IGF-II B chain, truncated IGF-II B chain, and a novel peptide derived from the E domain of the open reading frame of the IGF-II precursor gene (Dull et al., 1984). The existence of such a peptide has been postulated but never before observed. To determine whether this peptide has IGF-II activity, we have synthesized the complete molecule and are now purifying the crude product preparatory to testing it in our growth factor assay. We are also using the synthetic product to produce specific antibodies in order to develop sensitive assays for the determination of serum IGF-II peptide levels. These studies should further our understanding of how these growth factors are processed, how they function and what role they may play in the pathogenesis of endocrine disorders.

Reference:

1Department of Biomedical Sciences, University of California, Riverside.

110. Structural Analysis of Mouse Cerebellar Antigen L1

David B. Teplow, Melitta Schachner1

Neurons in the developing mouse cerebellum express a membrane protein(s) termed L1. Antibodies specific for this antigen inhibit the normal migration of cerebellar neurons from the external granular layer to the internal granular layer. This interference is thought to be a consequence of the disruption of normal cell-cell interaction between the migrating neurons and Bergmann glia, supporting cells of the cerebellum. To help understand this developmental process and its perturbation, we have continued our studies of the primary structure of the L1 antigen(s).

In previous studies we obtained N-terminal sequence information from L1 polypeptides of apparent molecular weights of 200,000, 140,000, and 70,000. We now have produced peptide fragments from each of these chains by digestion with Staphylococcus aureus V8 protease. The peptides were purified by reverse phase HPLC on a Vydac diphenyl column and loaded directly on a gas phase protein sequenator.

The peptide maps obtained through HPLC and the sequence information subsequently derived have helped us better understand the structural relationships among the L1 polypeptides and between them and other proteins. We have also designed oligodeoxy-nucleotides to be used in gene cloning. This combination of protein structure analysis and gene cloning should allow us to elucidate the complete primary structures of the L1 gene(s), message(s), and protein(s), and the function(s) of this protein in cerebellar development.

1Institute for Neurobiology, University of Heidelberg, Heidelberg, West Germany.

111. Modern Methods for the Chemical Synthesis of Biologically Active Peptides

Stephen B. H. Kent, Ian Clark-Lewis, Ruedi Aebersold, Karen Parker

Improved methods worked out over the past decade (Kent, 1980; Kent and Clark-Lewis, 1985), have formed the basis for the development of a peptide synthesizer that for the first time fully automates the process of assembly of protected peptide chains (Kent et al., 1984a,b). In the past year, improved synthetic technology based on this instrument has been developed in our laboratory. The process of chain assembly has been optimized by us using a double coupling procedure with chemistry adjusted for each amino acid residue so that any peptide chain can be synthesized at the rate of 16 amino acid residues per day with yields ranging from 99-99.8% per residue. Synthesis and monitoring problems associated with the incorporation of histidine residues have been resolved with the routine use of Boc-His (DNP). We have also found Trp(formyl) to be effective in eliminating modification of the indole ring during synthesis. Final HF deprotection conditions have been investigated to optimize yields of
Novel Envelope Proteins of Hepatitis B Virus: Biological Significance, Improved Diagnostics and Vaccines

Stephen B. H. Kent, A. Robert Neurath 1, Karen Parker, Chin Sook Kim, Nathan Strick 1

We have shown that two previously unrecognized proteins are present in the envelope of the hepatitis B virion (Neurath et al., 1984). These two proteins contain the immunodominant epitopes of the virus. Using protein chemical, immunochemical, and synthetic peptide techniques, we have located these immunodominant epitopes of the virus between residues 12-32 and 120-145 of the pre-S region of the env gene open reading frame (Neurath et al., 1985a). Synthetic peptides corresponding to these sequences are extraordinarily antigenic and immunogenic and mimic the epitopes with high fidelity (Kent et al., 1983). The peptides can be used as the basis of novel immunoassays to detect anti-virion antibodies; the anti-peptide antisera can be used in novel immunoassays to detect the hepatitis B virion. There is a strong, early immune response in humans to both these epitopes in the course of hepatitis B infection; this immune response is the earliest marker of HBV infection (Neurath et al., 1985b). The 120-145 epitope induces a T-cell response and can convert non-responder strains of mice to responders to other regions of the viral envelope proteins (Milich et al., 1985; Neurath et al., 1985c). Both epitopes are involved in attachment of the virus to hepatocytes—the primary step in the infective process. Existing HBV diagnostics do NOT detect the virus-specific epitopes of the virion and therefore cannot distinguish non-infective from infective carriers. The existing hepatitis vaccine licensed in the United States, which is prepared from the sera of infected humans, does NOT contain the immunodominant epitopes of the virus and does not stimulate an immune response to them. Trials in chimpanzees have shown that antibodies to a synthetic peptide corresponding to Pre-S (120-145) are virus-neutralizing (R. Prince, S. Kent, N. Strick and A. R. Neurath, in preparation). We have concluded that existing hepatitis vaccines should contain the full range of epitopes present in the virion and that "second generation" vaccines should contain all biologically significant HBV envelope protein epitopes. Currently, we are using several novel techniques to precisely locate (to a resolution of a single amino acid) the two immunodominant epitopes. We will investigate the antigenicity and immunogenicity of small peptides and rationally-designed analogs. Further diagnostics are being developed and additional neutralization studies of synthetic vaccine formulations are under way.

References:
The amino acid sequence of human epidermal growth factor (EGF) has homologies to both human and murine epidermal growth factor (EGF). Complete conservation exists in the positions of all of their six cysteines. αTGF binds to and possibly exerts its biological activities through interaction with the cell-surface receptor for epidermal growth factor.

We have chemically synthesized 0.5 mmole of human αTGF on PAM-resin using an automated peptide synthesizer (Kent et al., 1984). A double-coupling protocol was used throughout the synthesis. Following thiolysis of DNP protecting groups on histidines, peptides were cleaved from the resin and the remaining protecting groups removed using a low/high hydrogen fluoride procedure (Tam et al., 1983). The yield of target peptide was 78% as judged by preview sequence analysis of resin-bound protected peptide on a gas phase protein sequencer.

The synthetic αTGF was refolded in the presence of a mixture of oxidized and reduced glutathione in guanidium chloride. 125I-EGF competition binding to purified EGF receptor, αTGF-induced EGF receptor tyrosine kinase, and soft agar growth promotion assays are under way to characterize the activity of the synthetic αTGF. These results will guide future attempts to understand the structural origins of the biological activity of αTGF by the systematic design and synthesis of αTGF analogs and antagonists.

References:

Application of Chemically Synthesized Peptides in Biological Research
William D. Nelson, Ron J. Sweet, Suzanna J. Horvath

We have chemically synthesized a large number of peptides and conjugated to carrier proteins (KLH...
and/or ovalbumin). Our collaborators have used these peptide-protein conjugates to immunize rabbits or mice to give conventional heterogeneous antisera or monoclonal antibodies. Antibodies with the desired specificities are being used as biochemical reagents. We have prepared 27 peptides to raise antibodies against T-cell receptor gene products (Frank Fitch, University of Chicago and Patrick Kung, T Cell Sciences, Inc.), seven peptides to raise antibodies against MHC gene products (Steve Hunt and Iwona Stroynowski) and four peptides to raise antibodies against the homeobox of *Drosophila* (Carl Parker and Matthew Scott, University of Colorado).

In addition, we have synthesized 12 peptides to correspond to differing regions of the lysozyme molecule in our attempts to map out the epitopes with which T cells can bind the lysozyme molecule (Nilabh Shastri).

Finally, we have synthesized a whole series of active domains that permit us to study the function of these polypeptide chains, including the procoat protein of M13 (73 residues) and the Hin DNA-binding protein of *Salmonella* (31- and 52-residue peptides). We have been working on the synthesis of the γδ resolvase, a DNA-binding protein (25- and 43-residue peptides) and the complete homeobox of *Drosophila* (a 63-residue polypeptide). These projects have been done in collaboration with John Tomich, John Richards, Melvin Simon, Thomas Steitz, Carl Parker and Matthew Scott.

116. Improvements in Chemical DNA Synthesis

*Robert J. Kaiser, Jr.*

The automated chemical synthesis of DNA oligonucleotides with defined sequence has offered new approaches to the understanding of a variety of biological processes. Our current technology features the use of phosphite chemistry via activated deoxy-nucleoside phosphoramidites on a solid support. This method is characterized by excellent repetitive coupling yields, thus affording oligonucleotides of high purity in good overall yield. Automation of the procedure has insured rapid production of DNA. The advent of controlled pore glass as the solid support phase has allowed for the synthesis of long oligonucleotides.

Although the chain assembly process is quite efficient, other reactions in the synthetic cycle can lead to sometimes substantial loss of the product DNA. This is especially true for long oligonucleotides (>20 or so bases). Most notable of these side reactions are: 1) acid depurination; 2) phosphorylation/alkylation of nucleoside bases; and 3) loss of growing chains due to hydrolysis from the support by basic impurities throughout the synthesis cycle. We are currently investigating remedies for these problems.

The first two side reactions are best reduced by changing the nature of the protecting group(s) on the DNA bases. The amidine protecting groups developed by Carruthers *et al.* (1984) are being tested in this regard. The third side reaction may be overcome by changing the nature of the chemical linker joining the oligonucleotide to the support. A new, more resistant linker has recently been reported (Sproat *et al.*, 1985) and may prove to be useful. Other potential improvements are also being examined.

**References:**


117. The Chemical Synthesis of Amino-derivatized Oligodeoxyribonucleotides

*Lloyd M. Smith, Robert J. Kaiser, Jr.*

We have further developed the chemistry for introducing one or more aliphatic amino groups into synthetic oligodeoxyribonucleotides. These groups can readily be reacted with a variety of electrophilic functionalities to allow covalent attachment of other chemical species to the DNA in a controlled fashion. Our main thrust at this time is to use these amino groups as points of attachment of fluorescent dye molecules, using the subsequent fluorescent-labeled oligonucleotides as primers in DNA sequence analysis based on fluorescence detection. We also are examining the use of amino-containing oligonucleotides as ligands for coupling to solid supports for affinity chromatography, and in the production of synthetic restriction enzymes by attaching EDTA-like metal chelators. A variety of other possible applications exist as well.

The strategy for the incorporation of amino groups is to synthesize the 3′-phosphoramidite of an appropriately protected amino-nucleotide analog. This
phosphoramidite may then be incorporated into synthetic DNA oligonucleotides using the standard solid phase phosphite chemistry. Currently, our efforts have been directed in two areas: the introduction of a (single) terminal amino group at the 5' end of an oligonucleotide using protected 5'-amino-5'-deoxythymidine, (Smith et al., 1985) and the introduction of (potentially multiple) internal amino group(s) at the 2' position via protected derivatives of 2'-amino-2'-deoxyuridine.

These phosphoramidites are compatible with our Applied Biosystems automated DNA synthesizer, and indeed all have been successfully used on this instrument. Thus, we now have the capability to rapidly, cleanly, and automatically synthesize DNA oligonucleotides containing any desired number of reactive amino groups at virtually any point in a sequence. As such, this capability represents a powerful tool in the study of nucleic acids.

Reference:

118. Automated DNA Sequence Analysis Using Fluorescence Detection
Lloyd M. Smith, Jane Sanders

A prototype DNA sequenator has been constructed. This instrument uses an argon ion laser to excite fluorescence from bands of DNA synthesized in enzymatic sequencing reactions from a fluorescent primer oligonucleotide. The laser irradiates a 1 ul volume of a polyacrylamide tube gel in which the fluorescent DNA fragments are being electrophoresed in order to separate them by size. When a band of DNA molecules enters the area of the gel illuminated by the beam, fluorescence is emitted. This light is collected by a single fast aspheric lens passed through an interference filter to eliminate scattered excitation light and measured with a photomultiplier tube. The sensitivity and resolution of this system allows peaks to be detected up to 400 bases from the primer oligonucleotide. Four different dyes have been used that have different emission spectra; all four give adequate sensitivity in sequencing reactions. Reactions prepared using one specific dye for a particular sequencing reaction may be combined and electrophoresed together on a single tube gel. The color of the emitted fluorescence for any given band indicates the base that terminates the DNA fragment of that length. The data from this system are acquired and analyzed on an IBM PC XT computer. Software is under development that will automatically deduce the DNA sequence from the acquired data.

119. Automated Maxam/Gilbert Sequencing

A machine is under development that will automate the Maxam/Gilbert chemical degradation DNA sequencing reactions. In this machine the DNA to be sequenced is covalently attached to a solid support. It is then subjected to the chemical degradation reactions using the well-established valve technology employed in the gas-phase protein sequenator, the DNA synthesizer, and the peptide synthesizer. The DNA is eluted from the paper after the reactions are complete. The DNA may then be analyzed by conventional gel electrophoretic methods and autoradiography.

120. Application of Chemically Synthesized Deoxyoligonucleotides in Biological Research
Marilyn G. Tomich, Christina A. Hill, Suzanna J. Horvath.

Working in collaboration with many colleagues here at Caltech, more than 700 synthetic deoxyoligonucleotides have been prepared and have played important roles in a large variety of research projects. These include deoxyoligonucleotide probes that related to analyses of T-cell receptor genes, MHC genes, antibody genes, the PDGF receptor gene, and homeobox genes. Deoxyoligonucleotide probes also have been synthesized to work on projects related to in vitro mutagenesis of enzymes, RNA splicing in yeast, the enzymology of rearrangement in prokaryotes, the developmental biology of sea urchins and Drosophila, the molecular biology of Togavirus and a variety of other projects. Total synthesis of genes for the blue copper protein, plastocyanin (300 base pairs) and the serine protease, α-lytic protease (600 base pairs) have been accomplished by the improved synthesis of relatively large (40-65 bases) gene fragments and with a novel strategy for gene assembly (D. M. Perez, S. Boyle-Iverson, and J. H. Richards, unpublished results).
The ability to synthesize short DNA primers with high purity very rapidly was instrumental in establishing the primer-directed DNA sequencing method (Strauss et al., 1985).

We have been working on the improved synthesis and purification of relatively large (10-50 mg) amounts of deoxyoligonucleotides for the use of structure studies, such as NMR studies, DNA-peptide co-crystallization, and X-ray crystallography.

Reference:

PUBLICATIONS


Associate Professor: Elliot M. Meyerowitz
Research Fellows: Karl J. Fryxell, K. Vijay Raghavan
Graduate Students: Caren Chang, Madeline A. Crosby, Mark D. Garfinkel, Christopher H. Martin, Peter H. Mathers, Robert E. Pruitt, David W. Ruff
Research Staff: Carol A. Mayeda, Rita T. Mundy, Elizabeth F. Noell
Laboratory Staff: Robin K. Wilson

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Summary: Our work is directed toward understanding the molecular mechanisms of organismal development. We wish to discover how individual cells recognize their physical and temporal locations in developing organisms, and how the cells use positional and temporal information in the regulation of their genes. The organisms we use in our experiments are the laboratory fly Drosophila melanogaster and the weed Arabidopsis thaliana.

The Drosophila studies are concentrated in two areas. Our major effort is in the study of a single genetic region of the left arm of the third chromosome, the 68C puff. This region contains three coordinately regulated genes, expressed only in the third larval instar stage of development and only in a single tissue, the larval salivary gland. The genes code for three of the several polypeptides that are secreted by the larval salivary gland at the end of the third instar, at the time of puparium formation. These polypeptides comprise a glue that attaches the puparial case to its substrate for the duration of the pupal period. The work described in the abstracts that follow includes the study of factors that act in trans in the regulation of these genes, and the DNA sequences required in cis for interaction with the trans-acting factors. In addition, a genetic characterization of the 68C region of the Drosophila genome is described, and a series of experiments directed toward understanding the evolution of the 68C glue gene cluster are presented. The second set of Drosophila experiments is a study of gene expression in the development of the compound eye of the fly, with the goal of understanding the nature of the gene products involved in eye differentiation, and of the pattern in time and space in which they are expressed.

Our initial work with Arabidopsis, detailed in previous Caltech Biology Annual Reports, demonstrated that this plant is uniquely suited to certain types of recombinant DNA experiments because of its small (70,000 kilobase pairs) genome, its small size, ease of growth, and short generation time. This year we report a detailed analysis of genome organization in the plant, with the conclusion that there is remarkably little dispersed repetitive DNA in the nucleus. In addition, the cloning of seed-specific genes and of the alcohol dehydrogenase gene are described. The seed-specific genes, which may be hormonally regulated, are to be used in a study of tissue- and developmental time-specific gene regulation; the alcohol dehydrogenase gene will be used as a selectable marker in transformation experiments.

121. Factors That Regulate Glue Gene Expression
Peter H. Mathers

The glue genes at position 68C on the Drosophila third chromosome are expressed in a tissue- and time-specific manner and thus represent a model system for studying the factors that regulate gene expression in development. The steroid hormone ecdysterone and a product of the 2B5 locus on the X chromosome have been shown to be required for proper RNA production at 68C. Hansson and Lambertsson (1984) have shown that ecdysterone is required for initiation of glue gene expression. Crowley and Meyerowitz (1984) have shown that the hormone is also part of the signal that shuts off glue gene expression later in development. The work of Crowley et al. (1984) implicated a product of the 2B5 locus in the synthesis or stabilization of 68C glue RNAs. In an effort to extend these findings, work has been aimed at elucidating the effects of the
I(1)npr-1 mutation, at position 2B5, on 68C glue and total cellular RNA production and at determining the mechanisms by which hormone levels control gene activity.

Experiments with the I(1)npr-1 mutation, which is a non-pupariating lethal, show that mutant larvae fail to express Sgs-3, Sgs-7 and Sgs-8 RNAs in their salivary glands (Crowley et al., 1984). In addition, larvae carrying the I(1)npr-1 mutation appear to overproduce transcripts that are found in wild-type salivary glands immediately prior to glue induction. Thus it appears that a product of the 2B5 locus, which is nonfunctional in I(1)npr-1 mutants, is required for a developmental shift that results in repression of these early genes and induction of the glue genes at 68C. Because the period of glue expression has been termed the "intermolt" period, the genes responsible for the earlier RNAs have been named the pre-intermolt genes (Pig). One of these pre-intermolt genes is just 5' of the Sgs-4 gene at position 3C on the X chromosome and has been named Pig-1 (see Figure 1). These genes are divergently transcribed and separated by approximately 1 kilobase (kb) of DNA. Because Pig-1, like the other pre-intermolt genes, is salivary gland-specific in its expression, it is possible that Pig-1 and Sgs-4 share some common regulatory elements in the DNA that separate them.

![Diagram of 3C11-12 region](image)

**Figure 1.** Molecular map of the 3C11-12 region. RNA transcription units are represented by arrows showing the direction of transcription. BER and Hikone are deletions present in lab strains which eliminate and reduce Sgs-4 transcription, respectively, while leaving Pig-1 RNA levels unaltered from wild type. Dotted lines show uncertainty in the location of the ends of the Pig-1 transcript.

Work has also been directed at determining the level at which the glue genes respond to changes in the concentration of ecdysterone. By analyzing the production of newly synthesized transcripts from the 68C glue genes in the presence of cycloheximide, it has been shown that ecdysterone-induced repression of RNA accumulation does not require the production of intermediate protein products. These results suggest that the hormone (and probably its receptor) interacts directly with the glue RNAs or with the DNA at 68C, as has been suggested by the work of Gronemeyer and Pongs (1980). A similar analysis of ecdysterone-mediated RNA induction is currently in progress.

**References**


122. The Sgs-3 Gene is Controlled by Two Separable Regulatory Regions

**Madeline A. Crosby**

Using P factor-mediated transformation experiments, I have attempted to delimit the region required in cis for the correctly regulated expression of the Sgs-3 gene. It is possible to distinguish between mRNA and protein produced by introduced Sgs-3 genes and resident Sgs-3 genes by using different wild-type size variants. As reported previously (Biology 1983, Abstract No. 63; Biology 1984, Abstract No. 83), 2.3 kilobases (kb) of 68C DNA sequences upstream and 1.1 kb downstream of the gene allow production of abundant levels of Sgs-3 mRNA and protein in the appropriate tissue and stage. When a small fragment containing only 130 bases pairs (bp) of 68C DNA 5' of the Sgs-3 gene was used in transformation experiments, no expression from the introduced Sgs-3 gene was observed.

For the experiments described above, the Oregon-R size variant of Sgs-3, which produces an RNA of approximately 1100 nucleotides, was used in the transformation plasmid and the Hikone-R variant, which produces two RNAs of approximately 950 and 1000 nucleotides, was carried in the recipient *Drosophila* stock. On an autoradiogram of an RNA blot, a low level of expression from the introduced gene might be obscured by the intense signal from the resident genes. This might be the case for the pGA2.4 transformants, those described above as containing 130 bp of 68C DNA upstream of the Sgs-3 gene. The Sgs-3 gene from the wild-type strain Formosa produces an mRNA of approximately 800 nucleotides which is more easily separated from the Oregon-R variant on an RNA blot.
I performed genetic crosses to replace the resident Hikone-R Sgs-3 genes in the transformed stocks with the smaller Sgs-3 size variant from Formosa. This allowed detection of a low level of expression from the transformants obtained with pGA2.4, reduced approximately 10 to 40 fold from normal levels. An RNA blot of the salivary glands and carcasses of transformed animals at different stages revealed that the introduced Sgs-3 genes are still expressed in the appropriate tissue and at the appropriate time. There is also a very low level of expression observed in the salivary glands of tan prepupae, a stage at which Sgs-3 is not normally expressed.

Thus it appears that the regulatory region controlling the expression of the Sgs-3 gene consists of two separable parts: one part which is necessary for abundant levels of expression is located between 130 bp and 2300 bp upstream of the gene, and a second part which confers tissue and stage specificity is located within 130 bp 5' of the gene or within the gene itself. It is possible that the expression observed in the pGA2.4 transformants is affected by flanking DNA from the transformation vector. I am currently analyzing transformants obtained with a transformation plasmid similar to pGA2.4 in that it contains the same fragment of DNA from the 68C region, but which was constructed with a different transformation vector.

123. Studies on Sgs-3 Gene Regulation using lacZ Fusion Constructs

K. Vijay Raghavan, Rita T. Mundy, Elliot M. Meyerowitz

In the previous year's report we stated that we had started embryo injections with an Sgs-3/lacZ fusion gene to get germline transformation in flies. That construct had about 2 kb of 5' noncoding sequence present. We now have three independent transformation events with this fusion gene.

The transformants make the appropriate size fusion mRNA as viewed on RNA blots. The salivary glands also stain for β-galactosidase activity as viewed histochemically. Experiments to detect activity of the E. coli enzyme outside the pupal case were not successful. Some of the possible reasons for this are: a) the fusion protein is not secreted; or b) the protein is secreted but is inactive when secreted. The normal "glue" is cross-linked upon secretion. If the lack of staining histochemically is due to the fusion glue being cross-linked, agents that disrupt this (e.g., 2-mercaptoethanol) could restore enzyme activity. This is being tested.

We histochemically examined salivary glands from transformant males that also carried the mutation l(l)npr-1. These glands, unlike control siblings that were l(l)npr-1*, do not stain blue. This indicates that the fusion gene is regulated by l(l)npr-1 in the same manner as the unaltered Sgs-3 gene.

Experiments in this laboratory (Crosby and Meyerowitz, 1985) have shown that 130 bp of sequence 5' to the mRNA start site is sufficient for correct tissue- and stage-specific expression of the unaltered Sgs-3. We are currently injecting a Sgs-3/lacZ fusion that has the same 130 bp sequence 5' to the mRNA start site. We have also begun site-directed mutagenesis of Sgs-3 to identify the sequences that confer tissue and stage specificity.

Reference:

124. Sequences Required for Regulated Expression of the Sgs-8 Gene

Elliot M. Meyerowitz, K. Vijay Raghavan

The 68C glue gene cluster has three genes in it, each a structural gene for one of the glue polypeptides coded at 68C. We know that the only sequences required for normal regulated expression of the Sgs-3 gene are those within a couple of thousand base pairs of the gene. To find if the cis-acting DNA sequences required for normal time and tissue of expression of the Sgs-8 gene include any of those required for the coordinate expression of the nearby Sgs-3 gene, or, if the two genes are independently regulated, we have fused an E. coli β-galactosidase gene downstream of the sgs-8 signal peptide. Upstream of the fusion gene are the sequences normally found upstream of the Sgs-8 gene, downstream are 268 base pairs of sequence normally found downstream of the sgs-8 coding DNA. No sequences required for normal Sgs-3 expression are present. When this construct is transformed into flies, both the fusion RNA and protein with E. coli β-galactosidase activity are made in third larval instar salivary glands. Thus, Sgs-8 regulation is independent of Sgs-3 regulation, and the clustering of the 68C
are members of a related gene family (Garfinkel et al., 1984) have shown that the DNA site needed for this factor's regulation of the Sgs-3 gene is near that gene. If l(1)npr-1 is crossed into the Sgs-8/S-galactosidase fusion strain, neither the endogenous Sgs-8 gene nor the fusion gene expresses. Thus, the sequences through which the l(1)npr-1 product interact with the Sgs-8 gene are near that gene, and are different from the sequences through which Sgs-3 regulation is effected.

Reference:

125. Comparative Studies of Sgs-3 Glue Gene in Species Related to D. melanogaster

Christopher H. Martin, Elliot M. Meyerowitz

Studies of the three glue genes at 68C in Drosophila melanogaster have shown that these genes are members of a related gene family (Garfinkel et al., 1983). The largest of the three genes, Sgs-3, differs from the other two primarily by the presence of 37 tandem repeats of the five amino acids pro-thr-thr-thr-lys. The Sgs-3 gene also seems to have the control sequences necessary for tissue and time specificity located nearby (Crosby and Meyerowitz, unpublished).

Currently the Sgs-3 genes from three species of the melanogaster species subgroup, D. simulans, D. erecta, and D. yakuba, are being sequenced. There are two main goals of these studies. First, we would like to learn more about the evolution of the repeat region of the Sgs-3 gene. In addition, it is hoped that likely control regions can be found as highly conserved regions within a background of more rapidly evolving, non-essential sequences.

Preliminary analysis of these sequences has begun. The hydrophobic leader is moderately well conserved and the amino acid sequence following the repeats at the 3' end of the gene are highly conserved. In contrast, the consensus repeat sequence is markedly different at both the DNA and protein sequence levels. Thus, while each repeat unit in a given species conforms rather closely to one or two consensus sequences, the consensus sequence itself appears to evolve relatively rapidly.

The upstream sequences do appear to contain islands of relatively well conserved sequences. In addition, there are deletions of some upstream sequences in these species. These deletions should help to further localize any regulatory regions. In order to attach significance to any potential regulatory region, it will be necessary to have data from various functional studies of the upstream region. Such experiments are in progress in this and other labs.

Reference:

126. Use of the Inter-Species Gene Transfer to Analyze Glue Protein Gene Expression

Mark D. Garfinkel

Previous approaches to study the sequence requirements for Sgs-8 and Sgs-7 gene expression have made use of P factor-mediated transformation of the Drosophila melanogaster germine and hybrid test genes constructed in vitro from various constituent parts. In the case of the fusion between the Sgs-8 5' portion and the Sgs-7 3' portion, the hybrid gene probably directs transcription of an unstable or abnormal RNA species that has proved difficult to detect.

An alternate approach is to conduct germline transformation of Drosophila simulans. D. simulans is the insect species most closely related to D. melanogaster. The genetic maps (Sturtevant, 1929) and the polytene chromosome maps (Lemeunier and Ashburner, 1976) of the two species are nearly identical. Estimates of the time since speciation occurred vary from 1 million to 7 million years. Adults of the two species are almost indistinguishable anatomically. Both species are cosmopolitan in geographic distribution and in many locations are sympatric. Sterile hybrid progeny can be obtained in laboratory matings of the two species. These facts further support the notion that D. simulans is a reasonable heterologous host for testing D. melanogaster genes for function. Recently, a mutation in the D. simulans xanthine dehydrogenase (Xdh) gene was isolated. Defective P elements containing the D. melanogaster Xdh gene are capable of complementing the D. simulans mutation. When such P elements are mobilized by the complete P factor, germline trans-
formation of D. simulans was observed (Scavarda and Hartl, 1984). Their experiments provide a phenotypic marker for identifying transformation events in this species and evidence that D. simulans is a suitable host for assaying the function of D. melanogaster genes.

Molecular analysis (Meyerowitz and Martin, 1984) has shown that D. simulans contains DNA sequences similar, but not identical, to the 68C glue protein gene cluster of D. melanogaster. Restriction enzyme mapping of the two species' 68C chromosomal DNAs and variation of the conditions of genomic DNA filter washing showed that the glue protein gene clusters of these species differ at perhaps 20% of the base pairs. Similarly, I have found conditions of salivary gland RNA gel blot hybridization and washing that permit cloned radioactively-labeled DNAs homologous with Sgs-7 or Sgs-8 to react only with the RNA transcript from the same species; cross-hybridization to the other species' corresponding RNAs is either eliminated or greatly reduced.

I have constructed composite P elements with the D. melanogaster Xdh gene and unmodified glue protein genes from D. melanogaster. I am in the process of microinjecting D. simulans embryos in order to obtain transformants. Such transformants will be assayed for the expression of the D. melanogaster glue protein genes using stringent hybridization conditions. The series of experiments planned should enable us for the first time to analyze the in vivo cis-acting sequence requirements of all three 68C glue protein genes simultaneously, including Sgs-8 and Sgs-7.

References:

127. In vivo Footprinting at the 68C Locus
K. Vijay Raghavan

Recent developments in sequencing methods allow the direct sequencing of specific regions of genomic DNA and using these methods to footprint DNA-protein interactions in vivo (Church and Gilbert, 1984; Ephrussi et al., 1985). I am examining the 68C locus to see if any hormone-dependent binding of regulatory factors to the glue genes at this locus can be detected. I have constructed specific single-stranded probes which can be used to read sequences upstream of Sgs-3, -7 and -8. The regions for which the probes have been constructed have been decided on the basis of transformation experiments using these genes. I have so far been able to verify that these probes can be used to read sequences at the 68C locus and that this sequence matches the published sequence exactly.

Experiments currently in progress involve the isolation of salivary glands from larvae that are synthesizing glue mRNA and doing the dimethylsulfate (DMS) reaction on a) DNA extracted from glands; b) intact salivary glands; and c) intact glands treated with ec dysone. The salivary gland DNA is extracted from each of the above three sample sets, taken through the subsequent steps of the sequencing protocol for the "G" reaction in Maxam-Gilbert chemical sequencing, of which the DMS treatment is the first step, run on a sequencing gel, transferred to nylon membrane and probed with specific probes that allow the sequence ladder in the region of interest to be read. A comparison of the "G" reaction lanes from samples a, b and c will show if there is any protection or enhancement of specific regions due to binding of proteins. The specific holes or enhancements in the sequence would implicate these regions as the sites where regulatory factors bind.

Site-directed mutagenesis of these specific regions with P-factor transformation can then be used to examine the function of the regions that are revealed by pre-treatment with DMS.

References:

128. Lethal Mutations Flanking the 68C Glue Gene Cluster
Madeline A. Crosby, Elizabeth F. Noell

As reported previously, we have conducted a genetic analysis of the region flanking the 68C glue gene cluster by isolating lethal mutations uncovered by deficiencies that span this region. Three different mutagens were used: ethyl methanesulfonate (EMS), ethyl nitrosourea (ENU), and diepoxybutane (DEB). In the region from 68A3 to 69A1, 101 lethal mutations and nine visible mutations were recovered. We have
now completed our analysis of these mutants. The mutations proximal to 68C11 were assigned to smaller chromosomal intervals by deficiency mapping, but were not characterized further. In the region from 68A3 to 68C11, one visible mutation (rotated) and three lethal mutations [1(3)S17, 1(3)B76, and 1(3)v4-2] were previously known. We have isolated alleles of 13 new lethal complementation groups as well as new alleles of rotated, 1(3)B76, and 1(3)S17. Six new visible mutations from within this region were recovered on the basis of their reduced viability; all proved to be semi-viable alleles of lethal complementation groups. No significant differences were observed in the distribution of lethals recovered using the three different mutagens.

The cytological distribution of genes within the 68A3-68C11 region is not uniform. The region from 68A3 to 68A9 (6-7 bands) contains at least 13 complementation groups, two of which are complex; the adjoining region from 68B1 to 68C1 (4-5 bands) contains no lethals or any other known genes; 68C1 to 68C9-10 (8-10 bands) contains at least one lethal complementation group and the 68C glue gene cluster; and 68C9-10 to 68C11 (2-3 bands) contains at least two lethal complementation groups and the visible mutation rotated.

Each lethal was mapped on the basis of complementation with overlapping deficiencies, lethals that mapped within the same interval were tested for complementation, and the relative order of the lethal groups within each interval was determined by recombination. The developmental stage at which lethality is observed was determined for a representative allele from each lethal complementation group.

129. Sequence Studies of a Boundary Between Conserved and Non-conserved Sequences Near the 68C Glue Gene Cluster

Christopher H. Martin, Elliot M. Meyerowitz

A region near one end of the 68C glue gene cluster contains a boundary between rapidly and slowly evolving sequences (Meyerowitz and Martin, 1984). Initial indications of this came from observations of the melting temperatures of inter-species DNA fragment hybrids from either side of this boundary region. These studies showed that the 68C glue gene cluster was contained in the more rapidly evolving region.

Comparison of the restriction maps of the five species whose 68C glue gene region have been cloned has shown that the conserved region is at least 15 kb long. This region is not known to produce any RNA transcript. Also, an inversion breakpoint, HR15, lies within this region and is homozygous viable. The conserved region appears to be evolving primarily by base substitution and possibly small insertions/deletions. In contrast, the glue gene cluster region displays large insertions/deletions, inversions, gene duplications, and the appearance of repetitive elements.

These results limited the site of the boundary to about 1 kb. In an attempt to further characterize the boundary, this region is being sequenced in three species of the melanogaster species subgroup: D. melanogaster, D. erecta, and D. yakuba. Preliminary comparisons (between D. erecta and D. yakuba) reveal three regions. The first is the highly conserved region, which shows base substitutions and very small insertions/deletions. This is followed by an approximately 300 bp region, which is still well conserved, that contains several stretches of consecutive As and consecutive Ts. This is followed by a region that is noticeably less well conserved and contains larger insertions/deletions. It is hoped that these studies may yield some insights into the cause of this striking change in rate of evolution seen in a stretch of only a few hundred base pairs.

Reference:

130. Eye-specific Genes in Drosophila

Karl J. Fryxell, Rita T. Mundy

The compound eye of Drosophila provides an attractive system for studying neural development (Biology 1984, Abstract No. 90). We are interested in genes that are abundantly transcribed in specific cell types in the fly eye. We have concentrated our efforts initially on the rhodopsin gene(s).

A bovine opsin cDNA clone has been subcloned into the vector pSP64, and a 32P-labeled antisense bovine opsin RNA synthesized in vitro with SP6 polymerase. When this antisense RNA is used to probe an RNA blot,
it labels a single RNA species at the appropriate stringency. This RNA is poly(A)' and is found in adult fly heads but not bodies. Nick-translated bovine opsin cDNA is a much less effective probe, presumably because reassociation of a double-stranded probe limits its hybridization to very distantly related molecules. Screens of a cDNA library with a single-stranded RNA probe, however, proved to be difficult. More complicated cloning strategies, performed by Rubin, Pak and their respective colleagues, succeeded in retrieving the major opsin gene. We have used sequence information from these labs to construct synthetic oligonucleotides representing evolutionarily-conserved portions of the rhodopsin gene, and have used these oligonucleotides to retrieve clones from cDNA and genomic libraries constructed in this and other laboratories. We anticipate that this approach, as well as others in progress, will provide a complete inventory of the minor opsin genes of the fly in the near future.

We have also used the mutation Mc, which reduces the size of the eyes and optic lobes, to look for genes that are specifically expressed in these tissues. Of the "head-specific" genomic clones described by Levy et al. (1982), we find that seven out of the 17 clones tested are eye- or optic lobe-specific in this sense. We plan to use in situ hybridization to identify the cell types in which these genes are expressed.

References

131. Characterization of the Genome of Arabidopsis thaliana

Robert E. Pruitt

To characterize the genome of Arabidopsis, I have randomly selected 50 recombinant λ clones containing a total of almost 1% of the plant genome and analyzed their content of repetitive and unique DNA by various DNA blot, RNA blot and restriction digestion procedures. Some of the repetitive sequence families have been further characterized to give a better idea of the types of repetitive sequences present in the Arabidopsis genome. The degree of methylation of sequence in the plant has been examined using the methylation-sensitive restriction endonucleases Hpa II and Msp I. The conclusions that can be drawn include the following.

1) The nuclear genome, as represented in our recombinant library, consists almost entirely of unique sequences, with approximately one repetitive element interspersed every 125 kb.

2) The DNA that encodes the ribosomal RNAs constitutes the major class of cloned nuclear repetitive DNA. It consists of approximately 570 tandem copies of a heterogeneous 9.9 kb repeat unit.

3) There are an average of 660 copies of the chloroplast genome per cell. This makes it the major component of the repetitive sequences found in A. thaliana DNA made from whole plants.

4) The inner cytosine in the sequence CGGG is methylated more often than the outer cytosine in the tandem ribosomal DNA units, while very few differences in the methylation state of these two cytosines are detected in unique sequences.

132. Molecular Cloning of Arabidopsis Genes That Are Abundantly Expressed in Seeds

Robert E. Pruitt, David W. Ruff

In a previous report (see Biology 1984, Abstract No. 94), we reported the identification and molecular cloning of an Arabidopsis gene homologous with the 125 seed storage protein found in Brassica napus (Crouch and Tenbarge, 1983). We have since characterized this gene further by determining the direction of transcription and also by some preliminary DNA sequencing. DNA sequence data show that this gene is, in fact, homologous with the 125 storage protein gene of B. napus and encodes a very similar protein. Determination of the complete DNA sequence of this gene is now in progress.

In order to characterize what other genes might be abundantly transcribed in seeds, and, in particular, to identify the gene or genes that might encode a protein homologous with the 1.75 storage protein found in B. napus, we have identified several A clones from a genomic library that hybridize strongly with 32P-labeled cDNA made from poly(A)+ RNA isolated from embryos. Of the six clones examined so far, two are homologous with the large storage protein gene already cloned and overlap the region we had previously cloned. The other four clones appear to fall into three separate classes on the basis of their restriction maps, but could share sequence homology which is not detected by restriction mapping. When
each of these classes was used as a probe on an RNA blot containing seed RNA and leaf RNA, a single seed-specific RNA species was detected in each case. The 12S storage protein gene clones detected an RNA of approximately 1600 nucleotides as did clones from two of the other three classes. The final class detected an RNA that appeared to be approximately 750 nucleotides in length and since it is seed specific, it is probable that this represents a gene encoding the 1.7S storage protein.

Reference:

133. Cloning and Sequence Analysis of Arabidopsis thaliana Alcohol Dehydrogenase Gene

Caren Chang

We have cloned and determined the nucleotide sequence of a single copy gene from Arabidopsis thaliana likely to be the gene encoding alcohol dehydrogenase (ADH). The gene was isolated from a random recombinant library by cross-hybridization with a maize Adh1 gene probe. The DNA sequence contains an open reading frame capable of encoding a polypeptide the same length as maize ADH1 and ADH2 (379 amino acids), and having about 80% homology with both maize enzymes. This open reading frame is interrupted by six probable introns whose positions are conserved with six of the nine intron positions present in both maize genes (Figure 2). Sequences important for eukaryotic gene expression such as the TATA box, polyadenylation signal, and intron splice junction sequences are found in the expected locations. The deduced amino acid sequence is 47% homologous with horse liver ADH; conservation of the functionally and structurally important residues known for the horse enzyme further indicates that the Arabidopsis gene encodes ADH. Finally, the gene hybridizes to a specific, anaerobically-induced RNA in Arabidopsis whose appearance corresponds with the anaerobic induction of Arabidopsis ADH protein.

We will examine the function of this gene by testing its ability to restore the ADH activity in existing Arabidopsis ADH null mutants via Ti-plasmid transformation. Should this approach succeed, we hope to answer questions concerning the regulation of ADH in plants, and to develop Arabidopsis ADH as a dominant selectable marker for plant transformation.

Reference:

Figure 2. Highly conserved exons are split by introns that are identical in position but differ in sequence and in length. Three of the maize introns (IV, V, VII) are absent in the Arabidopsis gene. Due to the intron length differences, the Arabidopsis gene is much smaller than the maize ADH genes.


Professor Emeritus: Herschel K. Mitchell
Research Staff: Joan L. Roach

Support: The work described in the following research reports has been supported by the National Institutes of Health, USPHS.

Summary: At present and for the future I have no research staff, but I continue with the program of the past few years. This is concerned with studies on the regulation of differentiation using the unique system of cell hair morphogenesis in wing cells of Drosophila. This system was developed here over the last few years in collaboration with Dr. Nancy S. Petersen at the University of Wyoming. The collaboration continues at a distance and in the immediate future I am concerned with the purification of several proteins that appear to enter directly into structure during the multi-step process of construction of cell hairs by non-dividing cells.

Work on heat shock proteins in relation to phenocopy induction also continues.

134. Structural Proteins in the Morphogenesis of Cell Hairs

Herschel K. Mitchell

From earlier studies (Mitchell and Petersen, 1983), we demonstrated the resistance of two clearly defined time periods during which protein sheets and/or fibers are deposited in the construction of cell hairs. These processes are completed prior to cuticle deposition on cell surfaces. The first time period (27 to 35 hr into pupal life) involves construction of epicuticular in double and triple sheets external to the hair and cell membranes. This appears to be derived primarily from a protein of 50 kd in size and it may be the component given the name cuticulin by Locke (1966). The second time period (47-55 hr) appears to involve primarily a protein of 38 kd in size and this is deposited internally in hairs in bundles which give rigidity and final shape to cell hairs. Both of these protein components show multiple forms in isoelectric focusing, as does actin. Isolation of these proteins is in progress with the objectives of establishing their functions with certainty and using partial sequences to prepare probes for molecular studies.

References:

135. Spontaneous Degradation of the 70 kd Heat Shock Protein

Herschel K. Mitchell, Nancy S. Petersen1, Carolyn Buzin2

Much attention has been given to the induction of synthesis and the gene structure of the heat shock proteins (hsp) but little is known concerning the ultimate disposal of these components once they are produced in cells. We observed that the 70 kd hsp of Drosophila disappears in vivo in Drosophila larvae in a few hours at non-inducing temperatures. We and others also observed that the 70 kd hsp of Drosophila appears to be degraded in vitro on acrylamide gels following isoelectric focusing. This is in spite of the fact that the protein extracts and the gels are prepared under denaturing conditions. We have confirmed the fact that this degradation does indeed occur under a variety of denaturing regimens. The 70 kd hsp from a mouse cell line and a CHO cell line also undergoes degradation. We have presented extensive evidence
that the degradations observed are due to self-proteolysis. In addition we investigated the possible existence of other self-destructive proteins in differentiating wing tissue of *Drosophila* where little general proteolysis occurs. Thus, we have identified a number of non-heat shock proteins that also undergo spontaneous destruction. Each gives its own pattern of intermediate-sized peptides and each also undergoes degradation under non-denaturing conditions. It remains to be established whether these degradations observed *in vitro* also occur *in vivo*.

**Reference:**

1Department of Biochemistry, University of Wyoming, Laramie, Wyoming.
2City of Hope, Duarte, California.

**PUBLICATIONS**


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**Support (continued)**

National Institutes of Health, USPHS
National Science Foundation
Procter and Gamble

**Summary:** In the past year, we have made good progress in our understanding of signal transduction in both eukaryotic cells and in bacteria. Analysis of the genes that encode transducin has lead to the discovery of a new α subunit, designated Ta2. It shares with Ta1 the properties of being transcribed only in retinal tissue. It has approximately 70% amino acid identity with Ta1 and shows all of the properties of transducin α subunit, i.e., a distinct pertussis toxin modifiable site, a cholera toxin site, and conserved sequences that correspond to guanine nucleotide binding sites. The genomic fragments and the RNA transcripts corresponding to the two genes are different. We are currently exploring the localization of Ta2 protein and RNA and hope to determine the function in the retina of the Ta2 protein as distinct from the Ta1 protein. We have also completed the analysis of the sequence of the transducin γ gene product and the nucleotide sequence of a clone corresponding to a transducin β-gene subunit. The complexity of the G-protein system and the tissue-specific expression of a number of G protein-determining genes encourages us to continue the analysis. We hope to find out how specific G proteins are specialized to transduce signals from subsets of receptors found in differentiated cell types.

In the bacterial cell systems, we have concentrated on the analysis of specific transmembrane receptor-transducer molecules. We have designed and constructed a variety of mutants and cis-acting suppressors. We are studying the effect of these mutations on the structure of the transmembrane receptor and its ability to generate signals.

We are collaborating with Prof. H. Berg and his co-workers in the analysis of the behavior of cells carrying these mutant alleles and we hope to discover how protein receptors generate signals and how they modulate their own activity.

In this past year, we have made excellent progress in our studies on the mechanisms involved in regulating gene expression by recombination in the Hin-mediated inversion system. We were able to show that site-specific recombination is complex, requiring protein
factors in addition to the Hin enzyme. These factors are currently being purified. Furthermore, we found that a specific sequence corresponding to less than 60 base pairs is required to enhance the rate of site-specific recombination. This sequence can be anywhere on the same plasmid as the two recombination sites. Thus, it has properties that are analogous to those of the eukaryotic transcriptional enhancers. The mechanisms involved in the function of the recombinational enhancer are being investigated.

In our collaborative efforts with Dr. Alan Barbour, we have made a number of important discoveries in the past year relating to the mechanism of antigenic variation in *Borrelia hermsii*, the bacterium that causes relapsing fever. First, our work has clearly demonstrated that antigenic variation is the result of gene rearrangement. Furthermore, the genes or parts of the genes that encode the outer membrane protein of *borrelia* exist in both an expressed and non-expressed form on linear, extrachromosomal plasmids. Expression of a given membrane protein is the result of a site-specific recombination event on the linear plasmid that translocates the gene to be expressed and juxtaposes it to an appropriate expression site. The precise mechanism of this recombination event is currently being examined.

Finally, in the context of our attempts to develop a sophisticated system for studying and manipulating recombination in mice, we have continued to work on meiosis in the murine system and we have continued our development of gene mapping techniques using recombinant inbred strains of mice. In this past year, we collaborated with a number of workers and have succeeded in mapping genes that have been previously well-characterized neurological defects in mice.

136. Protein and Substrate Requirements for Hin-mediated Site-specific Recombination

*Reid C. Johnson, Michael F. Bruist, Moira B. Glaccum, Carol Lee, Melvin I. Simon*

The alternate expression of flagellin genes in *Salmonella* is the result of an inversion of a 995 base pair (bp) segment of chromosomal DNA. Previous genetic studies have identified the *hin* gene product as being absolutely required for this site-specific recombination event (Silverman and Simon, 1980). We have developed a cell-free system derived from *E. coli* capable of mediating inversions in the appropriate DNA substrate in order to study the reaction biochemically (Johnson et al., 1984). Toward this end, we have purified the 20,000 dalton molecular weight Hin protein to the point where it accounts for more than 50% of the total protein in the preparation. The Hin protein by itself is very inefficient at mediating recombination. The addition of crude extract derived from cells which do not contain *hin* restores the high rate of the reaction, indicating that an additional host component is required. Preparations of this heat-stable, proteinase K-sensitive host component have been purified over 200 fold and preliminary observations indicate that it may be a low molecular weight nucleic acid-binding protein of *E. coli*.

We also have been investigating the substrate requirements for the recombination reaction. The DNA must be supercoiled and the recombination reaction takes place within two 26 bp recombination sites, which must be in the correct configuration with respect to one another. Synthetic-derived wild-type and mutant recombination sites have been constructed to analyze the precise sequence requirements at the recombination site. Studies using these substrates have demonstrated that 1) the 26 bp sequence which structurally consists of two 12 bp imperfect inverted repeats is sufficient; 2) both inverted repeats (half sites) must be present at each recombination site and the correct spacing (2 bp) between the half sites must be maintained; and 3) complementarity but not the actual base sequence may be the critical feature at the center 2 bp of the recombination sites. In addition to the two recombination sites, a 60 bp sequence from the amino-terminal coding region of the *hin* gene is required. This recombinational enhancer increases the rate of recombination over 20 fold and can function at many different locations. It consists of two noncontiguous sequence domains whose relative orientation but not precise spacing with respect to one another is important (Johnson and Simon, 1985).

References:


137. Protein Binding of the Hin Recombination and Enhancer Sites

Michael F. Bruist, Reid C. Johnson, Melvin I. Simon

Phase variation in Salmonella typhimurium is the alternate expression of the two flagellin genes H1 and H2. This alteration is caused by the inversion of a segment of DNA in front of the H2 gene (Zieg et al., 1978). The Hin protein is responsible for the site-specific recombination reaction which causes the DNA inversion. Recently, we have developed an in vitro system for studying this reaction (Johnson et al., 1984) and have purified the Hin protein to better than 50% homogeneity.

Using the in vitro system, the conditions necessary for inversion have been defined. The DNA substrate must be supercoiled and contain two crossover sites (hix sites) in the proper relative orientation. A host function stimulates inversion but no other low molecular weight cofactors are required. Efficient recombination also requires the presence of an additional sequence of DNA, the inversion-enhancer site, which is normally located within the inverting segment of DNA (Johnson and Simon, 1985).

We have demonstrated that the Hin protein binds to the hix sites by showing that Hin protects the bases at the hix sites from cleavage by DNase I (DNA footprinting). The affinity of the Hin protein for the two hix sites is about the same and unchanged by the orientation of the inversion region. The binding to two sites on one DNA fragment appears to be independent.

We also have seen evidence for interaction of a Hin-associated protein with DNA at the inversion-enhancer sequence. A series of DNase I cleavage protections and enhancements is seen that defines two domains of interaction. The two domains are independent in that the binding at each one is seen in the absence of the other. However, if a small portion of one domain is deleted, the binding at that domain is entirely abolished. Loss of binding is accompanied by loss of inversion-enhancer function.

These experiments will help us to predict a mechanism for the site-specific recombination reaction produced by the combined action of the Hin protein, the inversion-enhancer sequence, and the host inversion function.

References:

138. The C-terminal Domain of the Hin Protein Is Responsible for DNA Sequence Recognition

Michael F. Bruist, Suzanna J. Horvath, Thomas A. Steitz, Melvin I. Simon

The Hin protein is related to another recombinase, the resolvase protein from the transposon γ-6. Like Hin, resolvase binds to a specific DNA sequence (res) as a part of its recombination reaction. It has been shown by protease cleavage that resolvase is composed of two domains, the smaller of which binds to the res site (Abdel-Meguid et al., 1984). This domain is composed of the last 43 amino acid residues of resolvase and is homologous to the last 52 amino acid residues of Hin.

We have demonstrated that an analogous domain does exist in the Hin protein and it binds to the hix sites. This was done by synthesizing a peptide at the Caltech Microchemical Facility that corresponds to the last 52 amino acid residues of the Hin protein as determined from the DNA sequence of the gene. Footprinting experiments show that the peptide protects all but the central bases at the hix sites from DNase cleavage. This implies that at least two molecules can bind to each recombination site and are responsible for the cleavage protection.

No cleavage protection or enhancement is seen for the peptide at the inversion-enhancer region; thus, it is probable that a different protein or a different portion of the Hin molecule is responsible for interaction with this sequence of DNA.

Currently, experiments are under way to determine if a smaller peptide will still recognize the hix sites. In addition, an effort is being made to co-crystallize the 52 amino acid peptide with a synthetic oligonucleotide containing the hix sequence. Such crystals will allow us to see the exact nature of the protein-DNA interaction responsible for recognition of the hix site by Hin.

Reference:
139. Molecular Basis of Antigenic Variation in *Borrelia hermsii*

Ronald H. A. Plasterk, Melvin I. Simon, Alan Barbour

*Borrelia hermsii* is a bacterium that causes relapsing fever in man. Each relapse is accompanied by the appearance in the blood stream of bacteria with a new surface antigen (serotype). Thus far, 26 different surface antigens have been identified. The switch between serotypes is associated with a rearrangement of structural gene sequences coding for the variable major proteins (VMP) (Meier et al., 1985). In order to determine the nature of the DNA rearrangements that are responsible for sequential activation of the different VMP genes, we made gene banks in bacteriophage λ of the different serotypes and selected the relevant clones. The picture emerging is as follows. Each serotype contains a set of untranscribed VMP genes. In addition, it contains a second copy of one of these genes that is fused to an "expression sequence." This gene is transcribed. Both the silent VMP genes and the expressed VMP gene are on extrachromosomal elements. The silent copies of the two VMP genes analyzed in detail are on "storage" plasmids of 28 and 32 kbp. The "expression" plasmid is 28 kbp. Surprisingly, it turns out to be a linear rather than circular DNA molecule; one of the ends is sensitive to degradation by Bal31 nuclease. On the basis of analysis of restriction maps and Southern hybridization, we propose that rearrangement and specific serotype expression may occur as a result of a single site-specific reciprocal recombination event between linear extrachromosomal elements.

Reference:

1 NIH Rocky Mountain Laboratory, Hamilton, Montana.

140. Functional Domains in Chemosensory Transducers

Kenji Oosawa, Melvin I. Simon

The chemosensory transducers in *E. coli* are transmembrane proteins that have three functions: 1) they bind specific attractant and repellent compounds; 2) they are involved in adaptation to ambient levels of attractant; and 3) they generate a signal that regulates the frequency of flagella reversal. The nucleotide sequences of three of the genes encoding receptor-transducer molecules have been determined. These are the Tar (aspartate) receptor, the Tsr (serine) receptor and the Tap receptor, which currently has not been assigned a function. On the basis of the amino acid sequences of these gene products, we developed a model for the disposition of the proteins with respect to the plasma membrane. There is a signal sequence that is not cleaved and acts as a first transmembrane region. The N-terminal region of the protein is situated in the periplasmic space and is involved in ligand binding. There is a second hydrophobic region that extends through the membrane and acts as a membrane anchor sequence and there is a cytoplasmic region of the molecule that is involved both in generating signals and in the adaptation function.

We are trying to answer questions about the mechanisms involved in transfer of information across the membrane from the N-terminal periplasmic region to the C-terminal cytoplasmic region of the protein, in addition to questions about the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling. We have used site-directed mutagenesis to make specific changes in amino acid sequence and to analyze the nature of the processes involved in signaling.

Reference:

141. Molecular Analysis of Meiosis in Mouse Testis

Kelwyn H. Thomas, Melvin I. Simon

The molecular mechanisms regulating the precision and specificity of meiotic pairing and recombination...
remain a matter of speculation and represent a problem of major biological significance. In mouse testis, these mechanisms comprise a series of well-defined sequential steps which are reflected in very distinctive cytological changes during successive stages of meiotic prophase. In an attempt to define the molecular events that regulate the early stages of meiosis, we have isolated poly(A)+ RNA from fractionated cells from a number of stages in meiosis. These sequences have been used to prepare cDNA libraries in λgt10. Differential hybridization has allowed us to isolate and identify cloned sequences that are expressed at specific stages early in meiosis. These clones are currently being characterized using the technique of in situ cytohybridization on frozen sections of testis from adult and prepuberal mice. We hope to be able to correlate specific transcripts with structural and functional features of cells that are undergoing meiosis. Antibodies specific to meiotic gene products are not available; however, they can be generated from DNA sequences using selected clones. These antibodies can then be utilized as fluorescent probes to localize proteins or enzymes to specific cell types. With utilization of expression vectors, sufficient overproduction of meiotic proteins can also be achieved to justify their use in functional studies.

142. The Genes Encoding the γ and β Subunits of Retinal Transducin and other G Proteins

Henry K. W. Fong, James B. Hurley, Rosemary Hopkins, Melvin I. Simon

The transduction of many hormone, neurotransmitter and sensory signals involves a family of guanine-nucleotide binding proteins. These related proteins serve to couple cell-surface receptors to functions that occur across the membrane and regulate the levels of intracellular cGMP, cAMP, and possibly other second messengers such as inositol polyphosphates, diacylglycerol and Ca²⁺. G proteins are a complex of three polypeptide subunits. To study the gene family of these regulatory proteins, we have isolated cDNA clones for the γ subunit of bovine retinal transducin and the β subunit, which appears to be identical to all G proteins.

We have learned that the γ subunit of transducin is a hydrophilic, 8,413 dalton polypeptide of 73 amino acids. The complete amino acid sequence confirmed that the subunit is not derived from a larger precursor transducin protein, but there is significant homology between the COOH-terminal region of transducin and the COOH-terminal protein and nucleotide sequence of the known ras genes. Transducin mRNA was found specifically in retinal cells and not in other tissues, implying that the γ subunits of other G proteins are different.

We also are studying the expression of the β-subunit gene. Transducin mRNA is found not only in retinal cells but in all other tissues examined, supporting the notion that the β subunit is identical in all G proteins and that G proteins have an important presence and function in every cell.

We hope to learn more about the function of the γ and β subunits of G proteins by the rational design of mutant genes in vitro and transfecting G protein genes into differentiated cultured cell lines.

143. Characterization of a Bovine Retinal cDNA Encoding a New, Transducin-like G Protein Subunit (Ta2)

Michael A. Lochrie, Ryn Miake-Lye, James B. Hurley, Melvin I. Simon

G proteins are guanine-nucleotide binding regulatory proteins that mediate the production of intracellular "second messenger" molecules in response to extracellular signals. For example, transducin is a G protein found in vertebrate rod photoreceptors that functions in the initial stages of the visual process by coupling photolyzed rhodopsin to the activation of a cGMP phosphodiesterase. Other biochemically well characterized G proteins include Gₛ and Gᵢ, which stimulate and inhibit adenylate cyclase activity, and Gₒ, the other G protein that is very abundant in brain tissue, but has an unknown coupling function. All G proteins have common features. They have three subunits: α, 39-45,000 daltons; β, 36,000 daltons; and γ, 8-10,000 daltons. The α and γ subunits differ among G proteins, but the β subunit appears to be shared by all. The α subunit binds GDP and exchanges this for GTP when activated by a ligand-bound receptor. The α-GTP complex activates proteins that regulate the levels of second messengers such as cAMP, cGMP, Ca²⁺, Mg²⁺, and inositol lipids. Hydrolysis of GTP to GDP by the α subunit resets the system to the pre-activated state. The ability of G proteins to bind and hydrolyze guanine nucleotides in a cyclic fashion
provides a mechanism for greatly amplifying the initial signal. The α subunits can be ADP-ribosylated by two bacterial toxins that use NAD⁺ as a cofactor. Ta and Gαa are modified by cholera toxin. Ta, Gαγ, and Gαβ are modified by pertussis toxin. Pertussis toxin modification prevents receptor/G protein interactions so as to maintain the system in an "off" state while cholera toxin inhibits GTP hydrolysis to maintain an "on" state. The β and γ subunits can modulate the activity of the α subunit.

Based on the amino acid sequences of the α subunit of transducin (see Biology 1984, Abstract No. 101), mixtures of oligonucleotides were synthesized by Dr. S. Horvath and used as hybridization probes to screen a bovine retinal λgt10 cDNA library provided by Dr. J. Nathans of Stanford. One clone was isolated that has a 1.7 kbp insert and a complete Gα protein coding region. However, this clone does not encode the α subunit of transducin (Ta1), but, instead a protein we currently call Ta2, which has several properties that are more similar to Ta1 than to any other known Gα protein. For example, only Ta1 and Ta2 have both a cholera toxin and a pertussis toxin modification site. Ta1 and Ta2 are abundantly expressed only in retina whereas other Gα proteins are expressed in a variety of tissues. Finally, partial amino acid sequences of Gαγ, Gαβ, and Gαβ, are very different from corresponding regions that are almost identical in Ta1 and Ta2. In spite of these similarities, the protein sequences of Ta1 and Ta2 differ in 69/350 positions. Most of these differences (44/69) are clustered in four short (10-20 amino acid long) regions in the amino terminus that are interspersed with highly conserved regions. The conserved regions are homologous to sequences in two other families of proteins that bind and hydrolyze guanine nucleotides—the Ras oncogene proteins and the translation initiation/elongation factors. Therefore, the constant regions probably form a nucleotide-binding site while the variable regions confer specificity of function. The α2 gene is a low copy number gene (1-5) and is highly conserved at least in mammalian species. An unusual feature of Ta2 expression is that the Ta2 cDNA is homologous to one 2 kbp RNA in mouse retina, but two very large 6-8 kbp RNAs in the bovine retina. Ta1 mRNA is about tenfold more abundant in the retina as a whole than is Ta2 mRNA. The receptor/effecter system that Ta2 functions in is unknown. Ta2 may be a cone transducin, a second rod transducin (e.g., one that inhibits the cGMP phosphodiesterase or leads to the production of other messages), or a neural or glial G protein. In the future, we hope to determine the retinal localization of Ta2 by in situ hybridization using nucleic acid and synthetic peptide antibody probes, study the biochemical properties of Ta2 by expressing the cDNA in E. coli or yeast or by purifying it from retina, crystallize Ta1 for structural studies, study the role of G proteins in developmental processes, determine if Ta1 and Ta2 are differentially expressed during development or if their expression is modulated in terminally differentiated cells, and isolate more Gα cDNA clones.

References:

PUBLICATIONS
The alphaviruses consist of about 25 members, many of which are important human or veterinary pathogens. Most of our work is done with the type alphavirus Sindbis virus, which is not pathogenic, but in collaboration with the National Institute of Health in Tokyo and the Centers for Disease Control in Fort Collins, Colorado, we also have conducted some comparative studies with a number of pathogenic viruses, such as Western equine encephalitis, Eastern equine encephalitis, Venezuelan equine encephalitis and Ross River viruses. The three equine encephalitis viruses are endemic in the United States, and as noted are an important cause of human and animal disease. Studies of these viruses are of significance not only for their intrinsic biological interest but also because of the health aspects.

We have obtained the complete nucleotide sequence of the RNA genome of Sindbis virus, some 11,700 nucleotides, and from this deduced the details of the organization of the genome and the amino acid sequences of the encoded proteins. The virus, although encoding only seven proteins, regulates the production of these proteins in two different ways. A subgenomic RNA encodes the structural proteins, which are produced in large amounts, and one of the replicase subunits, called nsP4, requires readthrough of a stop codon for production. Thus, although small, the virus has fairly sophisticated regulatory pathways.

Comparison of the amino acid sequence of the replicase components of Sindbis virus with those of several plant viruses shows the surprising result that Sindbis, tobacco mosaic, brome mosaic and alfalfa mosaic viruses are all related. The nucleotide sequence data being obtained for many viruses now are revealing details of the evolutionary history of these agents.

We are also actively involved in sequencing temperature-sensitive mutants and other types of variants of Sindbis virus, such as variants selected to be resistant to monoclonal antibodies and variants with altered virulence, in order to correlate specific amino acid changes with well studied physiological defects or changes in virus maturation. This type of study is extremely useful in working out the functions of the
various domains of the virus proteins. We have also begun to express cloned DNA copies of the Sindbis RNA genome in the vaccinia virus vector system recently developed by B. Moss, E. Paoletti, and other workers. This system shows great potential in allowing us to dissect details of the virus life cycle as well as having potential for vaccine production.

We are also working with the type virus of the Flaviviridae, yellow fever virus. We work at Caltech with the 17D vaccine strain of the virus, but in collaboration with the Centers for Disease Control, we are also doing some comparative work with the virulent strain of yellow fever as well as with other types of flaviviruses. Flaviviruses, which include in addition to yellow fever such viruses as dengue, St. Louis encephalitis and Murray Valley encephalitis, are also important disease agents. St. Louis encephalitis is endemic in the United States and dengue has recently become quite active in Mexico and the Caribbean. In addition, the mosquito vector of urban yellow fever, Aedes aegypti, has recently reestablished itself in Mexico and is established in the southeastern United States so that the potential for new outbreaks of yellow fever exists.

We have completed the nucleotide sequence of the yellow fever virus genome and from this deduced the amino acid sequence of the encoded proteins. Comparison of the amino acid sequences of yellow fever virus proteins with those of Sindbis virus does not reveal any homologies, and it appears that the recent reclassification of flaviviruses as a separate family was justified, especially in view of the amino acid sequence homologies found between plant viruses and Sindbis virus. Using this sequence data we have begun an in-depth analysis of the yellow fever replication cycle and have begun to express yellow fever proteins in the vaccinia vector.

144. Structure of the Yellow Fever Virus Genome

Charles M. Rice, Edith M. Lenches, Janice Pata, Sean R. Eddy, Se Jung Shin, Rebecca L. Sheets

cDNA clones have been constructed that together contain the entire nucleotide sequence of the yellow fever virus genome (YFV, 17D vaccine strain). Clones ranging from 2-7 kilobases in length were aligned by restriction analysis and sequenced using the base-specific chemical cleavage method. Inspection of this sequence reveals a single, long open reading frame of 10,233 nucleotides, which could encode a polyprotein of 3411 amino acids beginning from the first methionine residue. Comparison of this sequence with amino-terminal protein sequence data for the structural proteins (see Biology 1983, Abstract No. 97) and several nonstructural proteins shows that the structural proteins are the first products encoded in this reading frame and the gene order is 5'-C-M-E-NV3-?-NV4-?-NV5-3'. The genome organization is shown schematically in Figure 1. In this figure we have proposed a new nomenclature for the nonstructural proteins.

The extreme 5'- and 3'-terminal sequences of YFV RNA are homologous to those found for a closely related flavivirus, West Nile virus (Wengler and Wengler, 1981), and the complement of the 5'-terminal sequence (equivalent to the 3' terminus of the minus strand) is related to the 3'-terminal sequence. This suggests that the viral replicase/transcriptase may have related recognition sites for (-) and (+) strand synthesis. In addition, the 3' untranslated region contains three tandemly repeated sequences. Such repeated sequences also have been observed in alphavirus, but computer searches do not reveal any extensive amino acid homology between these two virus families which are otherwise similar in virion morphology (although they assemble quite differently), genome size, and host range. Northern blot analysis of RNA from YFV-infected cells reveals the presence of genome-length single-stranded RNA as well as RNase-resistant, double-stranded RNA forms which can be denatured to genome-length RNA. Smaller discrete virus-specific RNAs also have been identified (although present at low levels compared to genome-length RNA), some of which appear to lack 5'-terminal sequences of the genomic RNA. The derivation and function(s) of these small RNAs are unclear—they could represent subgenomic mRNAs, defective interfering RNAs, or specific degradation products of genome-length RNA. Experiments to sort out these possibilities are in progress.

Future experiments include 1) a more detailed analysis of YFV virion structure, 2) isolation and N-terminal sequence analysis of the remaining YFV nonstructural proteins and structural protein precursors from infected cells, 3) production and
analysis of YFV-defective interfering particles, and
4) expression with cDNA copies of the YFV genome using the vaccinia virus expression system (see Abstract No. 145).

References:

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YELLOW FEVER 17D GENOME (10,862nt)

Figure 1. Organization and processing of proteins encoded by the yellow fever virus genome. Untranslated regions of the genome are shown as single lines and the translated region as an open box. The open triangle is the initiation codon (AUG), the solid diamond the termination codon (UAA). A new nomenclature for the proteins is used. This nomenclature, the nonstructural proteins are named in order of the appearance of their genes in the genome. NS1 is also called NV3, NS3 is also called NV4, and NS5 is also called NV5. In addition, protein prM, the precursor to the M protein found in the virus, was formerly called NV2. The single-letter amino acid code is used for sequences flanking assigned cleavage sites (solid lines). Two other potential cleavage sites are shown as dotted lines. Structural proteins, identified nonstructural proteins, and hypothesized nonstructural proteins are indicated by solid, open, and hatched boxes, respectively. A more complete description can be found in Rice et al. (1985).

145. RNA Virus Expression from Cloned cDNA—The Vaccinia System

Charles M. Rice, Young S. Hahn

We are developing several alternative schemes for expression of cloned cDNA or RNA that has been transcribed and/or modified in vitro (see Abstract No. 154). One such system involves the use of vaccinia virus (VV) as a vector (Mackett et al., 1982; Panicali and Paoletti, 1982). VV is a vertebrate virus with a large DNA genome (180 kb) and a host range that overlaps that of Sindbis and yellow fever viruses. The advantages of this system are as follows. 1) Genes of interest can be cloned adjacent to a strong, early VV promoter and are "rescued" by helper VV by homologous recombination in vivo within a non-essential region of the VV genome (the selectable tk gene is commonly used). 2) The recombinant progeny that result in the inactivation of the helper tk gene can be plated directly on selective media (for tk-) and further identified by plaque hybridization. 3) Stocks of plaque-purified recombinants can then be used to drive high levels of foreign gene expression in tissue culture or in whole animals. VV RNA transcripts are capped, polyadenylated, and not spliced—which is particularly useful for expression of RNA virus genes or cellular cDNAs with potential cryptic splice sites.
Appropriate plasmids have been constructed that contain cDNA copies of the Sindbis genome designed to express several different viral transcripts: 1) full-length Sindbis; 2) nonstructural region; 3) structural region; 4) replication competent nonstructural region; and 5) defective interfering RNA. Recombinant VVs containing these plasmids are being constructed and the expression of these transcripts in tissue culture will be examined with respect to RNA and protein synthesis (see Abstract No. 146) as well as their ability to encode Sindbis functions and serve as substrates for Sindbis RNA-dependent RNA replication and transcription. Similar experiments are in progress for yellow fever virus (see Abstract No. 144).

References:

146. Expression of Sindbis Virus Structural Proteins via Recombinant Vaccinia Virus: Synthesis, Processing, and Incorporation into Mature Sindbis Virions

Charles M. Rice, Christine A. Franke¹, Dennis E. Hruby¹

We have obtained a vaccinia virus recombinant that contains a complete cDNA copy of the 26S RNA of Sindbis virus within the thymidine kinase gene of the vaccinia virus genome. This recombinant constitutively transcribed the Sindbis sequences throughout the infectious cycle, reflecting the dual early-late vaccinia promoter used in this construction. The Sindbis-derived transcripts were translationally active, giving rise to both precursor and mature structural proteins of Sindbis virus, including the capsid protein (C), the precursor of glycoprotein E2 (PE2), and the two mature envelope glycoproteins (E1 and E2). These are the same products translated from the 26S mRNA during Sindbis infection, and thus these proteins were apparently cleaved, glycosylated, and transported in a manner analogous to that seen during authentic Sindbis infections. Using epitope-specific antibodies, it was possible to demonstrate that recombinant-derived proteins were incorporated into Sindbis virions during co-infections with monoclonal antibody-resistant Sindbis variants. These results suggested that all the information necessary to specify the proper biogenesis of Sindbis virus structural proteins resides within the 26S sequences, and that vaccinia may provide an appropriate system for using DNA molecular genetic manipulations to unravel a variety of questions pertinent to RNA virus replication.

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147. Assessment of Neomycin Resistance as a Dominant-selectable Marker for the Selection and Isolation of Vaccinia Virus Recombinants

Christine A. Franke¹, Charles M. Rice, Dennis E. Hruby¹

The antibiotic G418 was shown to be an effective inhibitor of vaccinia virus replication when the appropriate concentration of drug was added to cell monolayers 48 hours prior to infection. Genetic engineering techniques were used in concert with DNA transfection protocols to construct vaccinia virus recombinants containing the neomycin-resistance gene (NEO) from the transposon Tn5. These recombinants contained the NEO gene linked in either the correct, or incorrect, orientation relative to the vaccinia virus 7.5 K promoter, which is expressed constitutively throughout infection. The vaccinia recombinant containing the chimeric NEO gene in the proper orientation was able to grow and form plaques in the presence of G418, whereas both wild-type vaccinia and the recombinant with the NEO gene in the opposite polarity were inhibited by more than 98%. The effect of G418 on viral growth may be mediated at least in part by a selective inhibition of the synthesis of a subset of vaccinia late proteins. These results are discussed with reference to using this system—NEO resistance to G418—as a positive selectable marker to facilitate constructing vaccinia virus recombinants that contain foreign genes of interest.

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148. Expression of RNA Virus Genes via Vaccinia Virus: 3' Promoter Deletion Analysis of the Vaccinia 7.5 K Early Promoter

Charles M. Rice, Young S. Hahn, W. M. Hodges¹, Christine A. Franke¹, Dennis E. Hruby¹

Vaccinia virus has been successfully used as an expression vector for a number of foreign genes (see Abstract No. 145). Its advantages include a broad host range, accommodation of large inserts of foreign DNA,
and cytoplasmic transcription with no evidence of splicing. It is thus useful for expression of heterologous genes lacking introns, such as cDNA copies of RNA viruses that replicate in the cytoplasm. Besides the production of functional virus-specific protein products, this system could also be used to make transcripts in vivo, which in the presence of appropriate host and viral functions would serve as templates for RNA-dependent RNA replication and packaging. Such templates would presumably require proper 5' and 3' termini for efficient utilization by the viral replicase. In the present study, we have produced a series of 3' deletions around an early vaccinia transcription initiation site (found in the 7.5 K cistron upstream sequences) fused to the bacterial chloramphenicol acetyl transferase (CAT) gene and studied their effects via the accumulation of CAT activity and stable transcripts. A deletion to +13 nucleotides (relative to the major start for initiation of early transcripts) had little effect on either the level of CAT or CAT-specific transcripts. However, deletions to +3 and -7 drastically reduced the level of both the early transcripts and CAT activity and a deletion to -30 abolished early transcription originating in this region. The 5' termini of these transcripts have been mapped using both S1 nuclease and primer extension and the results indicate that proper initiation of early transcription can occur, although inefficiently, within the foreign (CAT) insert.

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149. Sequence Analysis of Three Sindbis Virus Mutants Temperature Sensitive in the Capsid Protein Autoprotease
Chang S. Hahn

Complementary DNA made to the region of RNA encoding the structural proteins of three complementation group C mutants of Sindbis virus, ts2, ts5, ts13, and of their revertants, has been cloned and sequenced. These mutants possess defects in the post-translational processing of their structural proteins at the nonpermissive temperature. By comparing the deduced amino acid sequences of the mutants with those of the revertants and with the parental HR strain of virus, all three mutants were found to have a single amino acid substitution in the highly conserved COOH-terminal half of the capsid protein which gave rise to temperature sensitivity. ts2 and ts5 were found to have the same lesion and thus represent independent isolations of the same mutant, whereas ts13 possessed a different change. Reversion to temperature insensitivity in all three mutants occurred by reversion of the mutated nucleotide to the parental nucleotide, restoring the original amino acid. It has been previously postulated that the capsid protein possesses an autoproteolytic activity, which cleaves the capsid protein from the nascent polyprotein during translation. Comparison of the amino acid sequence of the capsid protein with that of serine proteases leads us to hypothesize that His-141, Asp-147, and Ser-215 of the Sindbis capsid protein form the catalytic triad of a serine protease. This hypothesis is supported by the finding that the three temperature-sensitive lesions mapped all occur near these residues: ts2 and ts5 change Pro-218 to Ser and ts13 has Lys-138 substituted by lle.

150. The Sindbis Virus Capsid Protein Autoprotease: Site-directed Mutagenesis of the Proposed Catalytic Amino Acids and Possible Requirement for the E3 Region for the Activity
Chang S. Hahn

There is evidence that the precursor of the structural proteins of the alphaviruses is processed by an autoprotease activity that exists in the C-terminal domain of the capsid protein (Simmons and Strauss, 1974; Scupham et al., 1977; Aliperti and Schlesinger, 1978; Hahn et al., 1985). The contribution made by the E3 protein, or some portion of it, to the protease activity is unknown. An experimental strategy to study this has been devised that involves construction of plasmids to be used as templates for production of in vitro transcripts using SP6 RNA polymerase; these transcripts will subsequently be translated in vitro using a rabbit reticulocyte system. The efficiency of cleavage will be determined by SDS-polyacrylamide gel electrophoresis to examine the proteins synthesized. The initial construct contains the 26S cDNA downstream from the SP6 promoter with convenient restriction sites for linearizing the plasmid prior to in vitro transcription. Constructs that have extra amino acids just after the capsid-E3 cleavage site or that possess sequential deletions of various lengths in the E3 protein are being constructed so as to determine
the minimum region of E3 necessary for this viral proteolytic activity.

We have postulated that the Sindbis capsid protein is a serine protease in which His-141, Asp-147, and Ser-215 form the catalytic triad of the protease (see Abstract No. 149). To test this hypothesis, each of these amino acids will be replaced with other amino acids using oligonucleotide site-directed mutagenesis. The activity of the altered proteins will be tested in the in vitro transcription-translation system described above.

References:

151. Sequence Analysis of Antigenic Variants of Sindbis Virus

Ellen G. Strauss, William Newman1, James H. Strauss, Alan Schmaljohn2

We are currently trying to localize the epitopes responsible for virus neutralization on the E2 glycoprotein of Sindbis virus. A battery of monoclonal antibodies to Sindbis virus has been isolated and characterized as to hemagglutination inhibition, virus neutralization, and glycoprotein specificity. Using a subset of anti-E2 monoclonal antibodies, which neutralize the infectivity of the AR399 (parental) strain, virus variants resistant to these antibodies have been isolated. We are currently in the process of sequencing the region of the genome encoding E2 in these variants to localize the neutralizing epitope. This can be done efficiently by sequencing intracellular RNA from Sindbis-infected cells directly by the dideoxy technique using synthetic oligonucleotide primers and reverse transcriptase. The changes that have been found to date are present in a restricted area of E2 encompassing about 40 amino acids, and all changes involve charged residues. This supports the idea that the major neutralization epitope of alphaviruses is formed by a comparatively small region of E2 that may have evolved specifically in response to pressures from the vertebrate immune system.

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152. Mapping of RNA− Mutants of Sindbis Virus

Young S. Hahn

Several temperature-sensitive (ts) RNA− mutants of Sindbis virus (RNA− mutants make less than 10% as much RNA at the nonpermissive temperature as the parental HR strain) have been isolated and classified by complementation into four groups (A, B, F, and G) (Strauss and Strauss, 1980). They are presumably defective in one of the nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) which are encoded in the 5′ terminal 7600 nucleotides of the genome and which function to transcribe a minus-strand copy of the genomic RNA and to transcribe both plus-strand genomic and 26S subgenomic RNAs from the minus-strand template. The presence of four complementation groups and four nonstructural proteins suggests that each complementation group defines mutations in one of the nonstructural proteins but no definitive evidence exists to support this hypothesis. We are trying to localize the ts RNA− mutations responsible for all of these complementation groups.

cDNA for one or more ts RNA− mutants in each of the four complementation groups (A: ts21,29; B: ts11; F: ts6,110; G: ts7,18) and their revertants was synthesized from a genomic 495 RNA template using as a primer a synthetic oligonucleotide complementary to the sequence near the 3′ terminus of the nonstructural region; this primer also had an end-labeled. Heteroduplexes were formed and the sequence near the 3′ terminus of the nonstructural region was determined by dideoxy sequencing. The RNA− mutations are mapped with respect to the 5′ region (2205 nucleotides) or BglII and XbaI for the 3′ region (5371 nucleotides). These fragments overlapped by 483 nucleotides. Gel-isolated ds cDNA fragments for the 5′ and 3′ regions were ligated with pBR322-derived vectors, Kahn 5.1 and pMT21, respectively. Proper cDNA clones (as screened by restriction analysis) have been analyzed by the Fisher and Lerman gel system (Fisher and Lerman, 1979, 1983) that can detect a single-base substitution based on differences in the melting behavior of duplex DNA molecules. Mutant and revertant cDNA clones were digested with restriction enzymes and the mutant fragments were 3′ end-labeled. Heteroduplexes were formed and the
fragments were separated according to size in a nondenaturing agarose gel followed by electrophoresis in the second dimension through a polyacrylamide slab gel containing a gradient of denaturant (urea and formamide) at 60°C. Once a mutation has been localized to a particular fragment, that fragment can be sequenced by the chemical sequencing method (or the corresponding region of the RNA can be sequenced using specific primers and the dideoxy chain-termination methods) to identify the nucleotide change(s) responsible for the ts phenotype in each RNA" mutant.

References:

153. The Synthesis and Processing of the Nonstructural Proteins of Sindbis Virus as Elucidated Using Antibodies

W. Reef Hardy

It is believed that Sindbis virus encodes four nonstructural proteins, which it uses to replicate and transcribe its single strand genomic RNA (Strauss et al., 1983, 1984). However, efforts to further characterize their structure and function have been largely unsuccessful for two reasons. First, they are produced in relatively small amounts relative to the structural proteins during infection. Second, the translation of the nonstructural proteins occurs early in the infection amidst a high background of host protein synthesis, thereby making the analysis of protein gel patterns complex.

One approach to this problem could entail the use of antibodies specific for the individual nonstructural proteins. From previous work in this laboratory, a cDNA copy of the complete Sindbis virus genome has been obtained and sequenced in its entirety, and tentative start points have been assigned to each of the virus nonstructural proteins (Strauss et al., 1984). Regions from within each of the putative genes for the nonstructural proteins have been cloned into an E. coli trp E fusion protein vector. Upon transformation of E. coli with these plasmids, sizable quantities of the N-terminal fusion products were produced. These fusion proteins were then used to immunize New Zealand white rabbits to generate specific polyclonal antisera to each of the nonstructural proteins. The utility of this approach has been demonstrated previously (Spindler et al., 1984).

At this time we have specific antisera against each of the nonstructural proteins and are using this antisera to immunoprecipitate these proteins from infected cells, which were pulse labeled at various times after infection followed by chases of varying duration. Protein processing in both wild-type virus and in mutants that are temperature sensitive in RNA synthesis and accumulate large polyprotein precursors at the nonpermissive temperature, is being studied in order to unravel the pathways of posttranslational processing of the polyprotein in vivo.

References:

154. Construction of Sindbis Virus Defective Interfering RNAs in vitro

Charles M. Rice, Henry V. Huang�

From comparative sequence analysis, it appears that at least three regions of the alphavirus 49S genomic RNA are important in the replication of the genome and transcription of the 3'-terminal subgenomic 26S RNA. Two of these regions, the 5' and 3' ends of the 49S RNA, are found in defective interfering (DI) RNAs (truncated and rearranged RNAs of viral origin that replicate only in the presence of a helper virus and thus compete with the helper genome for the replication machinery) and are thought necessary for production of full-length (-) and (+) 49S RNA. A third region (called the junction region), adjacent to the start of 26S RNA, may encode the recognition site for initiation of 26S transcription from the 49S minus-strand template. To study the replication and transcription of these RNAs, we have constructed cDNA clones of these three regions as well as clones containing full-length DNA copies of Sindbis virus 49S RNA, and several DI RNAs. We are attempting to reintroduce these viral sequences into tissue culture cells either as DNA or RNA and study their ability to replicate (or transcribe subgenomic
RNA) in the presence or absence of an appropriate helper virus. Along with current methods for in vitro mutagenesis, such a system will enable us to correlate structural features of the genome with their function in RNA replication and transcription.

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PUBLICATIONS


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Summary: We are interested in several fundamental aspects of cell biology. One problem we and others have come up against repeatedly is the need to figure out what a given gene product is contributing to cellular phenotype. For instance, many cloned DNAs have been selected for study based on an interesting pattern of expression during development, under different physiological conditions, or in a particular cell type. Study of cells or individuals severely diminished in or lacking the gene product of interest can be instructive in inferring function. For the past two years we have been exploring the possibility of using antisense nucleic acids as specific agents for establishing hypomorphic and null phenotypes. Stuart Kim began this work in the lab and has studied a model system for stable reduction of a gene product in animal cells. He also was able to investigate the molecular basis for the reduction in gene product. This work is described below. New members of the group, David Ruff and Roger Wagner, are continuing studies that further explore and apply antisense nucleic acids for the study of gene function.

Frank Preugschat and Paul Mueller are continuing
their work on two different aspects of RNA biogenesis and Craig Bond and Charles Reel have largely completed their studies of homologous recombination in animal cells.

155. Antisense RNA in Animal Cells

Stuart K. Kim

We have investigated the feasibility of using antisense RNA to diminish the level of a given gene product in a specific and stable fashion. The experiments are based on the idea that antisense RNA may be capable of hybridizing with mRNA (or its precursor) in vivo, rendering the messenger biologically inactive. As a test case, we have attempted to reduce thymidine kinase (TK) activity in cultured mammalian cells. Antisense TK RNA was expressed as part of a chimeric dihydrofolate reductase-antisense TK transcript. Genes coding for this RNA were introduced by DNA cotransformation into a mouse L cell line that expresses thymidine kinase constitutively. These transformants were then selected for progressively higher resistance to methotrexate provided by overproduction of dihydrofolate reductase (DHFR). The predominant mechanism of DHFR overproduction was gene amplification. The result of this selection procedure was a concomitant stepwise increase in intracellular antisense TK RNA concentration, owing to its presence on the same transcription unit as DHFR. In this manner cells expressing 5,000 to 10,000 antisense molecules per cell were obtained.

In several cell lines expressing high levels of antisense TK RNA, TK enzyme activity was reduced by 80-90% at high antisense RNA concentrations. One such line was further characterized at the molecular level, and it was found that RNA:RNA duplexes could be detected in the nuclear RNA fraction. In addition, sense TK RNA is present in these cells at a level equal to or greater than that observed in parental TK positive cells, but it is predominantly localized to the nucleus. These results suggest a mechanism for TK diminution in this cell line: antisense RNA hybridizes with sense TK RNA in the nucleus. These duplex-containing TK transcripts fail to enter the cytoplasm with normal efficiency, or are destroyed immediately upon arrival in the cytoplasm. As a consequence, the amount of TK messenger available for translation is reduced.

156. hnRNPs and RNA Transport

Frank Preugschat

One of the most basic processes that eukaryotic cells must carry out is that of transporting the products of transcription out of the nucleus to the cytoplasm. My main interest lies in defining some of the molecular components involved in the transport process. It has been recognized for a long time that precursor hnRNA is found complexed with proteins in the nucleus. Many of these proteins are confined to the nucleus and are not found complexed with mature mRNA in the cytoplasm. Functional roles in processing and transport have been postulated for these unique proteins. In collaboration with Dr. Gideon Dreyfuss and his colleagues at Northwestern University, cDNA clones corresponding to several of these proteins have been isolated from our human λgt11 library. One of these cDNAs codes for an isoform of the C or "core" protein, which is tightly associated with precursor hnRNA in the nucleus. By taking advantage of cross homology between the human C protein cDNA and the corresponding Xenopus laevis gene, I have isolated a prevalent Xenopus cDNA that putatively codes for the Xenopus equivalent to the human C proteins. I am currently characterizing the cDNA by DNA sequencing and I am also monitoring the expression of the gene at the RNA level. One of my objectives is to raise both polyclonal and monoclonal antibodies to an in-frame fusion protein. I will then use these antibodies to define whether or not the C proteins are involved in nucleocytoplasmic transport of RNA. I have set up a model system to study transport by using microinjected mRNA and the Xenopus laevis oocyte. Large amounts of pure RNA are synthesized in vitro with the SP6 transcription system, and I then inject the RNA into the large germinal vesicle of the oocyte. I am able to monitor RNA efflux from the nucleus through manual microdissection and I can measure turnover through RNA excess hybridization/RNase protection experiments. I will now use RNA substrates with various modifications in sequence content as well as Cap and poly(A) content to discover which features are critical for transport. I would like to monitor the association of microinjected RNA with Xenopus nuclear proteins, through the use of antibodies to Xenopus C protein. By microinjecting anti-C protein antibody into the
germinal vesicle, I hope to ask the question, "Does C protein have a functional role in RNA transport?"

An alternative experimental approach to this question will involve injection of antisense C RNA into oocytes and embryos to see what steps in RNA biogenesis, if any, are impaired as C-protein stocks are depleted.

157. Regulation of Mouse Metallothionein

Paul R. Mueller

We are interested in understanding how certain inducible eukaryotic genes are regulated. Our efforts have focused on the metallothionein gene of mouse, which can be induced by heavy metals. This induction is likely to be dependent on both cis-controlling elements (5' flanking regions of the metallothionein gene) and trans-controlling elements (some yet unknown transcription regulatory factor(s)). The metallothionein gene is being used as a model system to test a potentially general approach for describing some of the regulatory processes governing the expression of a given gene.

The 5' flanking region of metallothionein has been fused to the protein coding region of dihydrofolate reductase (DHFR) and we find that this renders DHFR inducible by heavy metals in cell lines transformed with this chimeric construction. DHFR levels have been quantitated by several methods: 1) measuring the ability of a cell extract to reduce folic acid; 2) binding H-methotrexate; and 3) viable cells producing various levels of DHFR can be identified and isolated by FACS (fluorescence-activated cell sorting) after incubation with a fluorescein methotrexate conjugate. The ease and flexibility afforded by these assays are being employed to address several questions regarding metallothionein regulation.

In one experiment, cell lines have been created in which the metallothionein/DHFR construct is amplified approximately 100 fold. This has been done by selecting cells resistant to increasing levels of methotrexate. These lines are being tested to see if the large number of metallothionein regulatory DNA sequences have titrated trans-acting regulatory factor(s). The titration effect is being monitored by evaluating the state of expression of endogenous metallothionein genes as the number of functional metallothionein promoters (driving DHFR) is increased. Preliminary analyses of this sort indicate that the Met I promoter is quite sensitive to titration of one or more factors.

158. Single-stranded Neutral Oligomers as Antisense Probes

David W. Ruff

Neutral oligomers offer several advantages for use in antisense regulation of genes. The neutral oligomers I am working with have phosphomethonate groups in their backbone instead of phosphate groups and, hence, are neutrally charged (Miller et al., 1983). Their neutral charge facilitates diffusion across membranes providing a potentially simple mode of introduction into the cell. They are highly stable in the cell since DNases and RNases cannot degrade the backbone and they form extremely strong and sequence-specific bonds to complementary sequences. We have worked out methods allowing us to label the oligomers with 32P, 35S, and 125I. Using purified, labeled oligomers, we have shown that when microinjected into Xenopus laevis oocytes the backbone is highly resistant to degradation. No detrimental effects have been observed on oocytes that have been microinjected with the oligomer. Experiments are now in progress to determine if various levels of a randomly selected neutral oligomer disrupt normal development when injected into Xenopus. If they are not generally toxic at the relevant doses, it is expected that they will provide a powerful tool for antisense-mediated reduction in specific gene expression. Their persistence in the presence of normal cellular nucleases suggest, in particular, that they may provide us with access to developmental stages and cell types in the embryo that are presently inaccessible due to turnover of injected antisense RNAs and conventional oligonucleotides.

Reference:

159. Homologous Recombination in Animal Cells

V. Craig Bond

The experiments described here address questions concerning recombination between complementing DNA fragments cointroduced into cells by DNA transfer. A model system using overlapping, truncated
fragments of the thymidine kinase gene was set up to test the physical parameters of recombination. It was observed upon transformation of these overlapping fragments into mouse L cells that the cells are able to rearrange these fragments into an intact, functional gene. Further, this can be done at a high frequency. The evidence suggested homologous recombination was being observed. Using this system, we examined a number of parameters that may affect recombination, such as linearization versus supercoiled substrates, location of the site of linearization in relation to the overlapping sequences, and the type of ends left by linearization (3',5' overhang, or blunt). Linearization of both partners within the region of sequence overlap was the single most important parameter facilitating recombination. Linearization anywhere else in either partner does not significantly increase the recombination frequency above that observed for the supercoiled form. Thus, location of the cut is key, and not the topological change from supercoiled to linear.

From the above experiments, we have begun to define a possible mechanism for how these molecules interact. It invokes double-strand nicking, or two close single-strand nicks of a supercoiled substrate, exposing ends. These ends are opened by breathing and chewback, or just chewback, exposing single-stranded regions of homology. These regions can anneal, the gaps are filled in and ligated shut. Key features of this proposed mechanism lend themselves to being tested. Breathing and exonuclease chewback, or just exonuclease chewback are required to expose the regions of homology. Therefore, the longer the region of homology, the more exonuclease chewback is required. This suggests that the recombination frequency is inversely proportional to the length of the region of homology. We are currently testing this aspect using truncated, cloned fragments of the hamster APRT gene with increasing regions of overlap from 7-1200 base pairs. Initial results support the exonuclease-repair mechanism.

160. A Search for Interchromosomal Homologous Recombination in Animal Cells

Charles Reel

The experiments described here were designed to investigate genetic recombination between independently introduced homologous gene sequences randomly integrated into the chromosomes of a mouse somatic cell line. Random chromosomal integration of each homologue facilitated the study of interchromosomal (chromosome–chromosome) reciprocal recombination and gene conversion among homologous genes.

Complementary herpes simplex virus thymidine kinase mutant gene/plasmid vectors with defined homology, pNEO3-5' 1.01 and pKPNFS-APRT, were constructed for use in independent transfections. Twenty-eight transformed mouse L cell secondary lines deficient in thymidine kinase activity (TK−) were established with these constructs at several integrated sequence copy levels. Transfection frequencies of from $2.5 \times 10^{-4}$ to $5 \times 10^{-5}$ were encountered for each line. Of these, selected lines were treated with the antibiotic mitomycin C, a mutagen causing nicks and breaks in double-stranded DNA, to see if recombination was enhanced. These and other lines (with and without mitomycin treatment) were then examined for evidence of TK activity resulting from homologous recombination. The results in examining $3 \times 10^8$ cells for TK activity indicate the absence of detectable productive recombination between the independently introduced and integrated TK gene sequences in the mouse genome. Previously reported frequencies ($10^{-4}$ to $10^{-5}$) for recombination between homologous TK sequences closely integrated in the same chromosome suggest that a sophisticated mechanism exists to prevent reciprocal recombination or gene conversion between sequences integrated into different chromosomes in mammalian cells. This finding has broad implications for the evolution of multigene families and gene regulation in animal cells.

PUBLICATIONS

CELLULAR BIOLOGY AND BIOPHYSICS

Howard C. Berg
Charles J. Brokaw
Scott D. Emr
John J. Hopfield
Elias Lazarides
Jean-Paul Revel
Ellen Rothenberg
Summary: Flagellated bacteria possess a remarkable motility system based on a reversible rotary motor linked by a flexible coupling (the proximal hook) to a thin helical propeller (the flagellar filament). The motor derives its energy from protons driven into the cell by chemical gradients or electrical fields. The direction of rotation of the motor depends, in part, on signals generated by sensory systems, the best studied of which analyzes chemical stimuli.

The rotation of the motor can be observed directly by fixing the filament to a glass slide; the cell body spins alternately clockwise (CW) and counterclockwise (CCW). These cells are called tethered cells. If chemical attractants are added to the medium surrounding tethered cells, chemotactic responses can be elicited. When the several motors of a free-swimming peritrichously-flagellated cell such as Escherichia coli turn CCW, the filaments work together in a bundle that drives the cell steadily forward (the cell "runs"); when the motors turn CW, the bundle flies apart, and the motion is highly erratic (the cell "tumbles"). Runs and tumbles occur in an alternating sequence, each run constituting a step in a three-dimensional random walk. When the cell swims in a spatial gradient of a chemical attractant, runs up the gradient are extended; this imposes a bias on the random walk that carries the cell in a favorable direction. Changes in concentration are sensed by specific chemoreceptors; CCW rotation is favored as more attractant is bound. We would like to understand how the motor works, what the signal is that controls its direction of rotation, and how this signal is processed by the chemical sensory system.

The spore-bearing stalk of the fungus Phycomyces grows away from solid objects. It is thought to have some kind of chemical radar. We are trying to learn how this system works.

161. Distance Dependence of the Signal Coupling Receptors To Flagella

Akira Ishihara, Jeffrey E. Segall

Bacteria respond to rising concentrations of attractant by increasing the extent of CCW rotation of their flagella. This response is mediated by receptors on the cell surface that bind attractant; it does not require transport or metabolism of this attractant. We have investigated the link between receptors and flagella by using iontophoresis to stimulate long, nonseptate cells. These cells were produced by using a cell division mutation or by growing cells in the presence of cephalaxin, a β-lactam antibiotic. Markers indicating the direction of rotation of their flagellar motors (ordinary polyhook cells fixed with glutaraldehyde) were attached with antihook antibodies (Ishihara et al., 1983).

Filamentous cells were stimulated iontophotically at different points along their lengths, and the response sizes at different markers were measured (Segall et al., 1985). We found no evidence for any long-range internal signal, such as a change in membrane potential. However, we believe that there is an internal signal, because the responses of markers near the end of a filamentous cell were larger when the pipette was near the cell body than when it was an equal distance away out in the external medium. The range of this signal is quite short, somewhere between 2 and 5 microns. Given that E. coli normally is only 1 to 2 microns long, a signal of longer range is not required. Indeed, it might be more important that the concentration of a chemical signal changes quickly to generate a rapid response. A likely candidate is a protein or ligand that is activated by the receptor and inactivated as it diffuses through the cytoplasm.

References:
162. Chimeric Transducers

*M. Patricia Conley, Tina Kramer*

Chemotactic responses of *E. coli* are mediated by integral membrane proteins known as transducers. In a collaboration with the laboratory of Prof. Melvin Simon, we are continuing to study the behavior of cells expressing genetically altered transducers, in an attempt to understand how these proteins translate information about receptor binding into signals that affect the flagellar motors.

In last year’s annual report, we described the behavior of strains expressing hybrid transducers, in which the N-terminal region of the Tar protein is fused to the C-terminal region of the Tsr protein. These fusions were made in the *tar* and *tsr* DNA at either of two conserved restriction sites. We found that the chimeric proteins were stable and functional and that functional domains inferred by the behavioral studies agreed with those predicted from genetic sequence analysis (Krikos et al., 1985). Functional domains for pH taxis were found to reside in both the N-terminal and the C-terminal portions of each transducer, with receptors for internal hydrogen ion (H+) mapping between the two restriction sites used for the constructions (Krikos et al., 1985).

Our more recent work has shown that the complementary set of hybrid transducers, namely those in which the N-terminal region of Tsr is fused to the C-terminal region of Tar, also behave in a manner expected if the transducers are built from discrete functional domains.

**References:**

163. Equilibrium Behavior of the Flagellar Rotary Motor

*Markus Meister, Shahid M. M. Khan*

We are continuing to test a model for the flagellar rotary motor (Berg and Khan, 1983; Khan and Berg, 1983) in which membrane-spanning particles linked to the cell wall via the S ring (the stator) exert force on proton-accepting sites at the periphery of the M ring (the rotor). The particles, which we call channel complexes, are assumed to have one channel (a proton-specific proton well) that conducts protons between one site and the external medium, and another channel (an aqueous pore) that conducts protons between the adjacent site and the cytoplasm. A complex moves at random (diffuses) along the periphery of the M ring, subject to constraints that depend on the occupancy of the proton-accepting sites. These constraints tightly couple forward motion of the channel complex to transfer of protons from the outside to the inside of the cell, and backward motion of the channel complex to transfer of protons from the inside to the outside of the cell, respectively. The motor normally operates near equilibrium, i.e., with the channel complex near a position (relative to the stator) at which the work that a proton can do in crossing the membrane, $e\Delta p$ (where $e$ is the proton charge and $\Delta p$ is the proton-motive force), is balanced by the work done against the elastic linkage, $fd$ (where $f$ is the time-average force exerted by the complex on the M ring, and $d$ is the distance between proton-accepting sites).

The variance in rotational speeds of tethered cells energized at protonmotive forces greater than about 30 mV indicates that these cells are free to execute rotational Brownian movement (Khan et al., 1983). The motor determines torque; it does not fix the position of the rotor relative to the stator. Our model is consistent with this behavior. At lower values of protonmotive force, there are some constraints on flagellar rotation. Cells repeatedly energized and de-energized stop at discrete angular positions, indicating a rotational symmetry of barriers to rotation of order 5 or 6. These barriers are evident when cells are gradually energized, but not when they are gradually de-energized. Once the motors spin rapidly, the barriers appear to melt (Khan et al., 1985).

Comparison of stall torques (deduced from the fixed angular displacements of tethered cells stalled by laminar flow) and running torques (deduced from the angular velocities of the same cells at zero flow) indicates that the flagellar motor always runs near equilibrium at speeds of order 10 Hz or less.

**References:**
164. The Flagellar Rotary Motor At Low Loads

Graeme Lowe

A fully-energized tethered cell is driven by a flagellar motor at a frequency in the range of 5 to 15 Hz. In this regime, the angular velocity is inversely proportional to viscosity; the torque is constant. The motor can be studied in a different regime by attaching small particles, e.g., polystyrene latex beads, to sheared flagellar stubs and watching them vibrate over a pinhole with a photomultiplier tube. Alternatively, vibration frequencies can be extracted by Fourier analysis of the scattered light. Cells in this regime appear to run their motors at constant angular velocity; the vibration frequencies do not change much as the external viscosity is raised by addition of Ficoll.

Using these methods on artificially energized cells, it should be possible to examine the temperature dependence of the torque generated by the motor at high speeds, under constant protonmotive force. The presence of a deuterium solvent isotope effect also will be tested. These effects are absent in tethered cells (Khan and Berg, 1983).

References:

165. Off-Line Analysis of the Motion of Tethered Bacteria

Steven M. Block, Andrew Nathan

Tethered bacteria are being used extensively in studies of energetics and behavior. Relevant data include the speed, duration and timing of the periods of clockwise (CW) and counterclockwise (CCW) rotation. The sheer volume of these data is noteworthy: cells of E. coli spin 10 Hz, reversing on average once per second. An experiment in which a microscopic field of 10 cells is recorded on video tape for one hour yields records of some 360,000 revolutions and 36,000 CW/CCW rotation intervals. Work continues on an instrument capable of digitizing the angular positions of video images of tethered cells in real time. The hardware, built by John Power in the Biology Division Electronics Shop, is up and running, and the software is about 80% complete.

166. The Avoidance Response in Phycomyces

Paul W. Meyer, Ivan J. Matus

A Phycomyces sporangiophore (spph) bends away from any object placed near its growing zone. For example, if in a closed observation chamber free of air currents, a clean, unused 22 mm diameter glass coverslip is placed 1 mm away from the growing zone, the spph bends away from the glass surface at the rate of about 1 to 2 degrees per minute.

The distance dependence of this avoidance response is very weak. A spph bends away from a coverslip at a distance of 4 mm about as fast as it bends away from one at 1 mm. The bending rate falls off rapidly at greater distances, declining by at least a factor of four between 4 mm and 6 mm. This result is not consistent with the simple model in which the spph detects objects by emitting a growth-promoting gas (e.g., water vapor, organic molecule) which then simply reflects off of the object; such a model predicts a 1/r to 1/r² distance dependence.

This result is consistent with our model described in the Biology 1982 Annual Report, in which the spph emits an inert gas, which is then converted to a growth-promoting gas (e.g., by oxidation) at the surface of the avoided object.

If this is correct, the magnitude and/or range of the avoidance response should depend on the chemical composition of the object; we now have evidence that a strong reducing agent, magnesium metal, elicits weaker avoidance responses than an inert material, such as copper or glass. Experiments are under way to test whether the rate of avoidance increases with the oxidation potential at the avoided surface and whether a weak adsorber such as boron nitride elicits weak responses.

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Summary: Evidence has accumulated indicating that the bending movements of cilia and flagella are generated by a sliding microtubule process, similar to the sliding filament process responsible for muscle contraction. Control mechanisms are needed to cause oscillatory bending, to maintain the phase differences between bending in different regions that are required for propagated bending waves, and to determine the parameters of particular bending patterns.

Our work makes particular use of ATP-reactivated movements of demembranated flagella as a source of experimental data, and of computer programs that simulate the movements of model flagella to relate theoretical mechanisms to experimental data. We hope to identify the types of control mechanisms that actually exist in flagella and cilia, to understand how parameters of movement such as frequency, wavelength, and bend angle are controlled, and to use this understanding to enable detailed study of the active process that generates sliding flagella and muscle. In the past year, experimental work has included the study of higher level control mechanisms involving regulation of motility by cAMP, calcium, and calmodulin. We hope that by exploiting the well-known biochemistry of these agents, we can obtain clues to the critical control points in the basic mechanisms of oscillation and bend propagation.

167. Calmodulin Removal from Demembranated Sea Urchin Sperm Flagella

Charles J. Brokaw, Sandra M. Nagayama

Exposure of demembranated sea urchin sperm flagella to millimolar Ca in the presence of Triton X-100 or other calmodulin-binding compounds, such as trifluoperazine, removes calmodulin from the flagella and sensitizes the flagella to exogenous calmodulin. Calmodulin removal has been quantitated by assaying the extracts by radioimmunoassay, by measuring phosphodiesterase activation, and by measuring the asymmetry-restoring effect on flagellar motility. These methods give reasonably similar answers, suggesting that about half of the flagellar calmodulin is solubilized as soon as the spermatozoa are demembranated with Triton, in the absence of calcium. A small fraction, perhaps 20%, of the calmodulin remaining in the flagellum is then solubilized by the Triton-Ca extraction. This work is continuing to determine whether the calmodulin removal that occurs under these assay conditions is equivalent to the calmodulin removal that occurs during motility observations.

168. Computer Simulation of Flagellar Movement

Charles J. Brokaw

Continued investigation of a simple flagellar model containing an active sliding mechanism with properties that cause it to generate an active shear moment that decreases with increasing sliding velocity has clarified the limitations of curvature-controlled flagellar models. This model will operate in the absence of viscous resistances with only a simple elastic bending resistance opposing the active sliding mechanism. This situation can also be solved analytically. The analytical solution demonstrates that a moment balance equation containing active shear moment controlled by curvature does not fully determine the movement of the model. At 0 viscosity, the details of the pattern of bend initiation at the basal end of the model determine the particular solution that is obtained. At higher viscosities, the requirement for 0 bending moment at the basal end of the flagellum becomes dominant, and causes the selection of preferred wavelength modes. Both of these factors can be overridden by specifying a control mechanism that requires the model to oscillate at a predetermined frequency.

These computations indicate that the specification of the control of active sliding by the local curvature of the flagellum is insufficient to fully determine the movement of a flagellum. Additional controlling factors, which have not yet been identified, are required.
169. Resact, An Oligopeptide from Sea Urchin Egg Jelly, is a Chemoattractant for Sea Urchin Spermatozoa

Gary E. Ward, Charles J. Brokaw, David L. Garbers, Victor D. Vacquier

Resact was initially purified from Arbacia egg jelly by virtue of its ability to activate the respiration and motility of Arbacia spermatozoa inhibited by low pH (6.5). It is a 14-amino acid peptide, which has been sequenced and synthesized.

A small droplet of resact solution injected into a suspension of motile Arbacia spermatozoa induces a rapid aggregation of motile spermatozoa in a typical chemotactic response. This response can be obtained with resact concentrations as low as 3 nM and is Ca-dependent. Similar concentrations of resact have been found to cause a rapid (<3 sec) down-regulation of the guanyl cyclase of the sperm flagellar membrane, but this effect is not Ca-dependent. Like other sea urchin spermatozoa, demembranated Arbacia spermatozoa respond to increased Ca concentration by increasing the asymmetry of flagellar bending.

The current model for sperm chemotaxis suggests that a decreasing resact receptor occupancy causes an opening of Ca channels in the flagellar membrane, an increase in internal [Ca], and a turn in the swimming path. Cyclic GMP and the membrane guanyl cyclase may be involved in an adaptation process that links resact receptor occupancy with Ca channel opening, but the details of this linkage remain to be clarified.

Surprisingly, we have been unable to observe chemotactic response of Strongylocentrotus purpuratus spermatozoa to speract, the analogous respiration and motility-stimulating peptide from S. purpuratus egg jelly.

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170. A Phosphocreatine Shuttle Mediates vivo Energy Transport in Sea Urchin Sperm Flagella

Robert M. Tombes, Charles J. Brokaw, Bennett M. Shapiro

Exposure of sea urchin spermatozoa to 1-fluoro, 2,4-dinitrobenzene (FDNB) in vivo causes an irreversible inactivation of flagellar creatine kinase activity. The response approaches saturation at about 10 µM FDNB. It can be measured by assaying enzyme activity, or by using demembranated spermatozoa to show that FDNB treatment inhibits reactivated flagellar motility of spermatozoa supplied with phosphocreatine and ATP in the presence of diadenosine pentaphosphate (an inhibitor of the adenylate kinase reaction: 2ADP + ATP + AMP) without inhibiting reactivated motility of spermatozoa supplied with ATP.

After inhibition with FDNB, intact spermatozoa swimming in sea water show an increased attenuation, or damping, of the flagellar bending waves as they propagate along the flagellum. This attenuation also becomes nearly maximal with 10 µM FDNB treatment, and in the 2-10 µM FDNB range, attenuation is seen with no decrease in flagellar beat frequency. The attenuation can be interpreted as a reduction in the rate of sliding between flagellar outer doublet microtubules, resulting from a reduction in ATP concentration. These observations are consistent with the idea that phosphocreatine is a major participant in energy transport by diffusion from the sperm mitochondrion along the length of the flagellum.

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171. Backwards Swimming Mutants of Chlamydomonas

Charles J. Brokaw, Rosalind L. Segal, David Luck

These mutants were identified by their failure to swim normally, and classified as mbo mutants: "move backwards only." It was initially thought that these mutants represented an aberration in the control mechanism responsible for switching between the normal asymmetric flagellar bending pattern that produces forward swimming and the symmetric flagellar bending pattern that normally produces backwards swimming when the cells are stimulated by light or other agents. Our photographic analysis of two of these mutants, recombined with the unflagellate mutation, unfl, to facilitate photography, suggests a different interpretation. mbo1 and mbo2 have distinct flagellar bending patterns, and neither one is identical to the normal symmetric flagellar bending pattern. Occasionally, these mutant cells are seen to undergo transient changes in bending pattern, as if they were making the spontaneous reversals that can be seen with wild-type cells. We have been able to photograph a small number of these events, and they reveal a transition to a normal reverse bending pattern.
pattern. Therefore, the mbo phenotypes appear to be a novel bending pattern that does not correspond to normal backward swimming.

The mbo mutants have a characteristic set of deficiencies in flagellar proteins and phosphoproteins; some of these are shared with another mutant, pf10, which has a very large amplitude bending pattern corresponding to the forward mode motility, and normal reverse mode bending patterns. Recombinants between pf10 and mbo mutants were examined, but we have been unable to constructively interpret the results: The motility phenotypes of the recombinant of pf10 with either mbol or mbo2 are similar, and are intermediate between the pf10 and mbo types of bending patterns. However, while the pf10 mbo2 recombinant shows all of the biochemical deficiencies of both parental types, the pf10 mbol recombinant shows none of these deficiencies!

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172. Effects of ADP on Waveform of Demembranated Sea Urchin Spermatozoa Reactivated at Low ATP Concentration

Edward F. Pate1, Charles J. Brokaw

The reactivated motility of demembranated sea urchin spermatozoa was photographed under two conditions: 1) with 20 µM MgATP, and 2) with 2 mM ADP in the presence of enough diadenosine pentaphosphate to inhibit formation of ATP from ADP, and enough ATP to overcome the competitive inhibition by ADP and produce the same beat frequency as in (1). Under these conditions, the bend angles of the flagellar bending waves were significantly greater in (2) than in (1). We interpret this to mean that states in the dynein cross-bridge cycle that retain ADP have different force-generating properties than states where the nucleotide binding site is empty. We are attempting to develop a detailed kinetic model of these effects.

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173. Movement Parameters of Trout Spermatozoa

Charlotte K. Omoto1, Charles J. Brokaw

Fish spermatozoa have been reported to have unusually high flagellar beat frequencies. We have attempted a complete characterization of the motility of spermatozoa from the steelhead trout (Salmo gairdneri) in order to see if high frequencies are associated with any unusual features of these flagella. Live trout spermatozoa are difficult to work with, because they normally swim for only 10 to 30 sec after activation by removal from the high-potassium environment in the semen. By working quickly, we have obtained photographs of these spermatozoa in the first few seconds after activation, and recorded beat frequencies in the vicinity of 60 Hz at 15°C. This is -50% greater than frequencies of sea urchin spermatozoa under comparable conditions. It may be possible because the flagella are short—30 to 35 µm instead of 40 to 45 µm for sea urchin spermatozoa—thus putting lesser demands on transport of energy substrates along the flagellum. Initially, the waveforms are similar to those of sea urchin spermatozoa, but they quickly change to a pattern characterized by much shorter wavelength as the frequency begins to decrease.

Reactivation of demembranated spermatozoa has been successful, but so far only the short-wavelength mode has been obtained, with frequencies as high as 80 Hz. Further examination of factors that determine the wavelength of the flagellar bending waves of these spermatozoa may be particularly informative.

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PUBLICATIONS

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Summary: Our laboratory has been interested in understanding how cells accomplish the accurate delivery of proteins from their site of synthesis in the cytoplasm to their unique subcellular organelle destinations. In all cells, different cellular functions are segregated into distinct membranes or membrane-enclosed organelles (e.g., mitochondria, nuclei, lysosomes, etc.). Each of these subcellular compartments contains its own unique set of proteins. Because these proteins are initially synthesized in the cytoplasm, the cell must possess mechanisms for the accurate sorting and delivery of these proteins to their correct organelle destinations. To ensure the fidelity of this process, proteins must contain signals in their sequence or structure that allow them to be properly distinguished. We have employed the yeast Saccharomyces cerevisiae and the bacterium Escherichia coli as model genetic systems to analyze the content of such sorting signals and to identify the cellular machinery responsible for deciphering these signals.

Yeast, like other eukaryotes, contains a variety of intracellular organelles. We have focused our attention on protein delivery to two of these: mitochondria and vacuoles. Greater than 90% of the proteins in mitochondria are coded for on nuclear genes. They are imported directly from the cytoplasm into mitochondria in an energy-dependent process. Many of these proteins are initially synthesized as larger precursors with transient amino-terminal amino acid extensions. These pre-segments are processed from the precursors after import by a protease within the mitochondria. During the past year, we have been studying import of the \( \alpha \)-subunit protein, a component of the mitochondrial F\(_1\)-ATPase. This protein is made initially as a precursor with an amino-terminal extension of approximately 20 amino acids. We have employed gene fusions to demonstrate that the pre-segment of the \( \alpha \)-subunit protein functions as a mitochondrial targeting and import signal that can direct heterologous proteins into mitochondria in vivo. We have also devised strategies for selecting mutants altered in their ability to assemble normal mitochondria. Characterization of these mutants, which is in progress, may allow us to identify components of the mitochondrial protein import machinery.

The yeast vacuole is a lysosome-like organelle that contains a variety of hydrolytic glycoprotein enzymes. Delivery of these proteins to the vacuole is mediated by a portion of the secretory pathway. Vacuolar proteins, like secretory proteins, transit from the cytoplasm into the endoplasmic reticulum where they become modified with N-linked core oligosaccharides. The proteins are then delivered to the Golgi complex where further glycosyl modifications take place. The Golgi is also the site where it is presumed protein sorting takes place. From this organelle, vacuolar proteins are transported to the vacuole and secretory proteins are packaged into vesicles that deliver the proteins to the cell surface. To address how the cell is able to distinguish between these two classes of proteins, we have constructed fusions of the yeast PRE\(_1\) gene, coding for the vacuolar proteinase carboxypeptide Y, to the SUC\(_2\) gene of yeast, coding for the normally secreted enzyme invertase. The PRE\(_1\)-SUC\(_2\) gene fusions code for hybrid proteins that exhibit invertase activity. Our results indicate that an amino-terminal segment of the CPY protein is sufficient to direct delivery of a CPY-invertase hybrid protein to the yeast vacuole. Also, we have selected mutants that are defective in vacuolar delivery of the CPY-invertase hybrid protein. In these mutants, the CPY-invertase hybrid protein as well as wild-type CPY protein are mislocalized to the cell surface. The mutants have allowed us to identify a number of genes in yeast that are required for normal sorting of vacuolar enzymes. Cloning these genes and defining the proteins they code for should enable us to better
understand how protein targeting and organelle assembly take place in yeast.

One such gene in yeast, SEC18, has been shown previously to be necessary for both protein secretion and vacuolar protein delivery (Novick et al., 1980). In a sec18 temperature-sensitive mutant, both secretory and vacuolar enzymes accumulate in the endoplasmic reticulum at the nonpermissive temperature. To better understand the role of the SEC18 gene product in protein transport, we have cloned this gene by complementation in yeast of the sec18 temperature-sensitive (ts) growth defect. In addition, antibody has been raised against a segment of the SEC18 gene product. This antibody is being used to define and localize the SEC18 gene product in wild type and sec18 mutant yeast.

Unlike yeast, E. coli does not contain intracellular organelles. However, it does possess a complex cell envelope consisting of an inner and an outer membrane as well as a unique aqueous compartment bounded by these two membranes, the periplasm. In this bacterium, exported proteins must be accurately targeted from the cytoplasm to one of these three extracytoplasmic compartments. In our studies, we have been analyzing delivery of the LamB protein to the outer membrane. LamB, like other exported proteins, is initially synthesized in the cytoplasm as a precursor protein with an amino-terminal amino acid extension. We have isolated mutants that are defective for export of the LamB protein. These mutants define components in the LamB signal peptide that are essential for normal export of this protein (Emr and Silhavy, 1982). Presently, we are characterizing suppressors of one of the signal sequence mutants that restore normal export of this protein. It is hoped that such second-site mutations will provide additional insights as to how this sequence functions in directing export of the LamB protein.

References:

174. Definition of a Mitochondrial Protein Targeting Signal

Jinnie M. Garrett

Most mitochondrial proteins are coded for by nuclear genes and synthesized in the cytoplasm of the cell. These proteins must be sorted from other proteins in the cytoplasm and accurately targeted to their unique destination within the mitochondria. Many mitochondrial proteins are synthesized as larger precursors with transient amino-terminal amino acid extensions that are processed after import into the mitochondrion by a protease in the internal matrix. In vitro import studies have shown that the pre-segment is required for protein import as processed precursors are not imported into the mitochondrion.

In order to define the amino acid sequence determinants necessary for the in vivo targeting and import of mitochondrial proteins, we have constructed gene fusions between the ATP2 gene of S. cerevisiae, which codes for the mitochondrial F1-ATPase a-subunit protein, and either the E. coli lacZ gene (codes for β-galactosidase) or the S. cerevisiae SUC2 gene (codes for invertase). The ATP2-lacZ and ATP2-SUC2 gene fusions code for hybrid proteins that efficiently target to yeast mitochondria in vivo. The mitochondrial-associated hybrid proteins fractionate with the inner mitochondrial membrane. Together, results obtained with these gene fusions and with targeting-defective ATP2 deletion mutants provide evidence that the amino-terminal 28 amino acids of the a-subunit protein precursor are sufficient to direct both specific sorting of this protein to yeast mitochondria and its import into the organelle. Currently, we are mutating this mitochondrial targeting signal in order to more accurately define the sequence components contained within the signal that allow for its specific recognition by mitochondria.

175. Characterization of Conditionally Lethal Mutants Affected in Mitochondrial Functions

Jinnie M. Garrett, Jane D. Goldsborough

We are studying the mechanism of mitochondrial protein import using gene fusions between the ATP2 gene (nuclear gene coding for the a-subunit of yeast mitochondrial F1-ATPase) and the E. coli lacZ gene. Hybrid proteins containing more than 200 amino acids of the a-subunit protein fused to β-galactosidase are
efficiently targeted to mitochondria and prevent normal functioning of the organelle. Cells expressing these hybrid proteins are no longer able to grow on non-fermentable carbon source, e.g., glycerol. This respiration-defective phenotype (Gly−) has been exploited to select Gly+ revertants. Mutants obtained in this selection were tested for normal levels of hybrid protein expression. Among such mutants, most have been found to contain nuclear mutations that are not linked to the ATP2-lacZ hybrid gene construct.

Many of the Gly+ mutants also exhibit a temperature-sensitive (ts) growth phenotype on all media tested. The ts and Gly+ phenotypes cosegregate in genetic crosses indicating that both phenotypes result from the same mutation. Presently, the mutations define five complementation groups. Initially, we are focusing our attention on one of the ts Gly+ mutants, M6. Closer analysis of the gene that is affected in this mutant should provide a better understanding of its role in mitochondrial functioning.

A yeast DNA clone has been identified that complements the ts growth phenotype of the M6 mutant. Chromosomal integration of this clone followed by gene mapping studies demonstrate that the clone contains the wild-type allele of the mutated gene in M6. Subcloning and DNA sequence analysis of this gene presently is in progress. Ultimately, we hope to define the gene product that is altered in the M6 mutant and determine its site of residence in the cell. This will be a starting point for additional biochemical studies of the role of this gene product in mitochondrial functioning.

176. Genetic Analysis of Protein Localization to the Yeast Vacuole

Vytais A. Bankaitis, Lianna Johnson

We are interested in the problem of how yeast localize proteins to their correct subcellular organelles. Specifically, we have been studying the localization of carboxypeptidase Y (CPY) to the yeast vacuole. Our goal is to elucidate the nature of the signals elaborated by CPY that facilitate targeting of this protein to the yeast vacuole. Furthermore, we wish to characterize elements of a cellular machinery that might interact with such signals, or might be otherwise involved in the vacuolar localization process. To this end, we have constructed yeast strains that synthesize hybrid proteins with amino termini derived from CPY and a carboxy terminus derived from the normally secreted protein invertase. Vacuole fractionation and immunofluorescence data indicate that relatively short amino-terminal segments of CPY are sufficient to redirect invertase to the yeast vacuole. We are currently attempting to define the minimum CPY sequence that functions in vacuolar targeting.

One phenotypic consequence of directing invertase to the yeast vacuole is the inability of such strains to utilize sucrose as a sole carbon source. This feature has provided us with a simple selection for mutants that secrete CPY-invertase hybrid protein to the yeast periplasm and thereby become phenotypically Suc−. Many such vpt mutants (for vacuolar protein targeting) have been obtained. All such vpt mutations are recessive to wild type, are trans-acting, and define at least eight complementation groups. We have demonstrated that vpt mutants exhibit a hybrid protein-independent accumulation of pre-vacuolar forms of wild-type CPY protein. Preliminary evidence also indicates a vpt-mediated defect in the proper localization of other wild-type vacuolar proteases as well. However, general protein traffic through the yeast secretory pathway appears otherwise normal. It seems that vpt mutants are specifically defective in vacuolar protein targeting and as such may be defective in protein sorting functions. We are currently attempting to clone the various VPT+ alleles so as to elucidate the roles of their corresponding gene products in the yeast protein localization scheme.

177. Identification of a Gene Product Required for Protein Secretion in Yeast

Kurt A. Eakle

A series of temperature-sensitive mutants that have defects in the process of protein secretion have been described in yeast (Novick et al., 1980). These mutants accumulate subcellular organelles analogous to the endoplasmic reticulum, Golgi apparatus, and secretory vesicles described in the secretion pathway for cells of higher eukaryotes. One mutant, sec18, at the nonpermissive growth temperature accumulates the yeast equivalent of endoplasmic reticulum and blocks protein transport to the Golgi apparatus. We are interested in characterizing the location and
function of the product of this gene.

The SEC18 gene has been cloned by complementation of the temperature-sensitive growth phenotype exhibited by the sec18 mutant. A large open reading frame contained within the SEC18 clone has been fused to the E. coli lacZ gene. By expressing this SEC18-lacZ gene fusion in E. coli, it has been possible to detect and purify a Sec18-galactosidase hybrid protein. Antiserum directed against this hybrid protein has been raised in rabbits. We are using this antiserum to 1) detect the native SEC18 gene product in yeast, 2) determine the level of expression of the SEC18 gene, and 3) determine the subcellular location of the Sec18 protein by immunofluorescence and subcellular fractionation. In addition, a more detailed analysis of the SEC18 gene is in progress, including DNA sequence analysis of the gene and construction of new sec18 mutant alleles.

Reference:

178. Defining an Essential Component Within the LamB Signal Sequence

Elliot Altman, Nancy Kaup

Previous analysis of 60 independently isolated mutants defective in the localization of LamB protein to the outer membrane of E. coli have defined four amino acids within the lamB signal sequence that are critical for efficient export of this protein (Emr and Silhavy, 1982). We have obtained 20 independently isolated pseudo-revertants of one of the lamB export-defective signal sequence mutants (lamB578) that are intragenically suppressed for the LamB localization defect (Emr and Silhavy, 1983). We are studying these in an effort to better understand the role of the signal sequence in directing protein translocation across the bacterial inner membrane. The intragenic suppression exhibited by these pseudo-revertants is exerted at the level of LamB export as evidenced by the restoration of some degree of proper localization of the LamB protein to the outer membrane. However, the pseudo-revertants define four distinct classes, based on their efficiency of suppression. Sequence analysis of the intragenic suppressor mutations should permit us to better define the role in export initiation of the four apparently critical residues within the signal sequence.

References:

1Undergraduate, Georgetown University.

179. Determining the Limitations of the Outer Membrane of Escherichia coli

Elliot Altman

It has become increasingly clear over the past few years that E. coli contains a complex export machinery to sort and deliver its noncytoplasmic proteins to their final cellular destinations. We observed that if wild-type LamB protein, normally produced at 100,000 molecules per cell and localized efficiently to the outer membrane, is overproduced some fivefold, using a multicopy plasmid to increase expression, cell death ensues. Yet if an export-defective LamB protein is similarly overproduced, the cells remain viable. We had originally reasoned that this overproduction lethality might result from the saturation of some essential component(s) of the E. coli export machinery. Biochemical analysis has shown, however, that the overproduced wild-type LamB protein is processed, sorted and localized to the outer membrane normally. Thus, the overproduction lethality occurs at the level of the outer membrane. We do not yet know, however, what the mechanism of this overproduction lethality is.

Two independently isolated mutants have been obtained that suppress the LamB-mediated overproduction lethality. Biochemical analysis has shown that the overproduced LamB protein is processed, sorted and localized to the outer membrane in the two mutants as efficiently as it is in wild-type E. coli, yet without any lethal effect. The mutants define two genetic loci mapping at 58 and 92-94 minutes, respectively, on the E. coli chromosome. Further analysis of the mutants will hopefully elucidate the nature of the overproduction lethality and indicate how the E. coli outer membrane can be modified to accommodate excess protein.

PUBLICATIONS


Professor: John J. Hopfield

Support: The work described in the following research reports has been supported by the National Science Foundation.

Summary: The general area of interest is the physics of how biological processes take place. We attempt to abstract from biology important chemical and physical properties, to understand, through theory and experiments, how these properties may come about in terms of structure, dynamics and context, and to then take these results back into biological systems to test our findings. Molecular areas in which studies have been made include the relationship between protein structure and local group chemical kinetics of heme proteins and the mechanism of biological electron transfer processes. The information processing of a network of neurons is also a physical process, and the "computational" behaviors of such nets can be approached in terms that make use of concepts from statistical mechanics. We attempt to relate such ideas to processing in simple biological systems and subsystems such as taste learning, a categorization in Limax maximus.

180. Emergent Properties of Neural Networks

David Feinstein$^1$, John J. Hopfield, Eric Mjolsness$^2$, John Platt$^1$

Emergent or collective properties of systems are behaviors which come about through the interactions of many similar objects, and which are not displayed or directly anticipated from the properties of very small systems. Large neural systems may display such emergent properties. The sense of the flow of time, the intactness of memories, and attention are plausibly emergent properties. We simulate systems of 30-1000 neurons to examine what biologically useful collective properties spontaneously arise. Several such properties do occur, including an ability to do content-addressable memory tasks and to "compute" answers to certain pattern recognition problems. Learning algorithms, the effects of unlearning, the dynamics of network decision-making, the relation of the model to more realistic properties of neurons, the use of such networks for pattern recognition, the generation of activity sequences, the effect of hierarchy in neuronal connections and physical circuit realizations of a model of collective activities are being pursued. Use of such ideas in electron circuit hardware of novel design is also being studied.

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181. Electron Transfer Processes

David N. Beratan$^1$, Alvin D. Joran$^1$, Peter Dervan$^1$, John J. Hopfield, Jose Nelson Onuchic$^1$, Burton A. Leland$^1$

Biological electron transfer reactions typically involve exchange of electrons over large distances (10-20 Å). These electron transfers are atypical chemical reactions because their rates are determined by very weak donor-acceptor interactions. A body of mostly untested theoretical ideas has evolved to relate the dependence of transfer rate on distance and
driving force. With these ideas in mind we have synthesized the family of molecules where the distance \( N = 0, 1, 2 \) can be varied. Exothermicity also can be varied by metal choice \( M \) or by peripheral substitutions on the porphyrin or quinone. Measurements of the transfer rates and lifetimes of the charge separated states are under way. Theoretical predictions of the dependence of rate on \( N \) have been made (see figure).

Conventional electron transfer theory is deficient in many areas. Work is in progress to explicitly include the structure of the intervening material (e.g., protein, or bicyclo[2.2.2]octane above) in the rate calculation. Electronic exchange is intimately coupled to the motion of surrounding bonds. In most chemical processes, the bond motions are fast compared to the time scale of electron transfer, but the motions of internal parts of the protein molecules may easily be on the same time scale. Theories including modes with such time scales for nuclear reorganization are being developed.

1Division of Chemistry and Chemical Engineering, California Institute of Technology.

**PUBLICATIONS**


**Professor:** Elias Lazarides  
**Senior Research Fellows:** Yassemi G. Capetanaki, W. James Nelson  
**Research Fellows:** John V. Cox, Bruce L. Granger, Horst Hinssen, Randall T. Moon, Matthias Staufenbiel, Catherine M. Woods  
**Graduate Students:** Lois M. Banta, John N. Ngai  
**Research Staff:** Adriana Cortenbach, Ilga Lielausis  
**Laboratory Staff:** Parvin Hartsteen  

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**Summary:** The major emphasis in our laboratory is to understand the mechanisms that underlie the expression, assembly, and topogenesis of various cytoskeletal domains during differentiation. As model systems we use avian and mammalian erythropoiesis, muscle and lens differentiation as well as neuronal differentiation. Over the past several years we have devoted a considerable amount of time defining and characterizing the various cytoskeletal domains in these cell types, as well as the mechanisms by which these domains become interconnected during terminal differentiation. Two of these domains on which we have focused our attention are the spectrin-actin network that assembles under the plasma membrane of red blood cells and intermediate filaments. In avian erythroid cells these two domains are interconnected and interlink transmembrane polypeptides to the nucleus. A major principle to emerge from our work thus far is that each of these two domains is
independently regulated in a cell type-specific and developmentally-regulated manner. They are subsequently joined with each other, also in a cell type-specific, developmentally-regulated manner. Additionally, the protein constituents of each of these two domains are also independently regulated in the same cell-specific developmental manner. Therefore, in totality the combinatorial events in the expression, composition, assembly and joining of these two domains are substantial during morphogenesis. For example, all components of the spectrin network are expressed constitutively during avian and mammalian erythroid development. However, expression of intermediate filaments is developmentally regulated at the transcriptional level in a positive manner (i.e., accumulation) during avian erythroid development and negatively (i.e., repression of expression and filament removal) during mammalian erythroid development.

Perhaps this pattern of expression is linked to the morphogenetic requirements of these two cell lineages in the two different species—retention of the nucleus in avian red blood cells and enucleation in mammalian red blood cells. In an analogous fashion, expression of the various subunits of the spectrin network is both tissue specific and developmentally regulated in a cell type-specific manner during differentiation. Having defined the tissue-specific patterns of expression of these polypeptides, the regulation of their assembly and their cell type-specific mechanism of joining, our long-term plan is to study the expression of these proteins at the gene level and set up transformation systems where we can manipulate and alter their tissue-specific patterns of expression and thereby study the consequences in cytoskeletal morphogenesis and cellular physiology.

182. Transfection of Murine Erythroleukemia Cells with the Chicken Vimentin Gene

John J. Ngai

Friend murine erythroleukemia (MEL) cells provide an excellent system in which to study the events of erythropoiesis in vitro. We have shown previously that the expression of the intermediate filament subunit, vimentin, is rapidly and extensively repressed during chemically-mediated differentiation of MEL cells (Ngai et al., 1984). This repression is primarily due to a decrease in vimentin mRNA abundance, suggesting that the regulation of vimentin expression in this developmental system resides at the level of transcription and/or RNA stabilization. In an attempt to dissect further the mechanisms involved in regulating vimentin gene expression through differentiation, experiments have been initiated to transfect MEL cells with a cloned chicken vimentin gene (Capetanaki et al., 1983). The major issues to be addressed include the following. 1) Can the chicken vimentin gene be expressed in a mouse cell environment? 2) If so, can the exogenous gene be regulated during MEL cell development as the mouse vimentin gene is? 3) Which of the two chicken vimentin mRNAs (which differ in the lengths of their 3' nontranslated regions due to the utilization of multiple polyadenylation sites) will be expressed in the mouse cell? The latter two points are particularly interesting, since we have observed a specific induction of the smaller vimentin mRNA during chicken embryonic erythropoiesis in the definitive cell lineage (Capetanaki et al., 1983). Hence, we wish to determine if MEL cells can both recognize and/or discriminate the chicken vimentin polyadenylation signals in the generation of 3' termini, and if they can utilize these signals in a manner analogous to their avian counterparts.

The initial strategy to carry out the above-mentioned experiments has been to co-transfect a cloned chicken vimentin gene in both phage and plasmid vectors and a cloned hamster adenosine phosphoribosyl transferase (aprt) gene into an aprC MEL cell line by calcium phosphate precipitation. Unfortunately, the frequencies of transformation are low by this method (<1 transformant per µg aprt plasmid per 10⁶ cells). Furthermore, the large size of the vimentin gene (>10 kb with flanking sequences) places a constraint on the maximum allowable vimentin:aprt molar ratio, and hence renders a lower than desirable frequency of co-transfection (4:1 vimentin:aprt molar ratio renders <50% co-transfection). Of six MEL cell lines harboring an intact chicken vimentin gene isolated thus far, only one appears to express vimentin mRNA at a very low level, as judged by RNase protection assays using SP6 polymerase-synthesized [32P]-labeled RNA probes. Experiments are now being carried out to construct hybrid plasmids containing both the chicken vimentin and the hamster aprt genes, and to use such plasmids
in subsequent transfection studies.

References:

183. The Major Neurofilament Subunit Gene Structure and Expression

Yassemi G. Capetanaki

Previous studies from this laboratory have shown that the genes for the intermediate filament subunits vimentin, desmin and glial fibrillary acidic protein (GFAP) are represented as single copies in the haploid chicken genome (Capetanaki et al., 1983, 1984). Furthermore, the tissue specificity and the developmental expression of these proteins reflect similar qualitative and quantitative patterns at the mRNA level (Capetanaki et al., 1983, 1984; Ngai et al., 1984). Hence, the expression of the above-mentioned intermediate filament subunits is regulated at the transcriptional level or by differential RNA stabilization but not at the translational level. The differential expression of keratins during terminal differentiation of epithelial cells also appears to be regulated at the mRNA level. Consequently, the obvious question at this point relates to the expression of the fifth class of the intermediate filament subunits, the neurofilaments. These filaments are composed of a 70 K core polypeptide (NF70) and two larger polypeptides in association with this core. The NF70 polypeptide was previously thought to exist only in neurons. However, it has been detected in the erythroid cells of chicken embryos and young chickens but is nearly absent from the erythroid cells of adults (Granger and Lazarides, 1983). Another interesting feature of these neurofilaments is the fact that they are markers and perhaps determining factors in the development of the central nervous system and may play a role in degenerative neurological disorders such as Alzheimer's disease. In order to examine the mechanisms of neurofilament subunit gene regulation during neuronal differentiation as well as during avian erythropoiesis and compare them with that of the other subunits, we have attempted to isolate cDNA and genomic clones for the NF70 subunit. Putative NF70 genomic clones have been isolated by cross hybridization with GFAP and desmin cDNA probes. At the same time, putative NF70 cDNA clones have been isolated by screening a agt11 cDNA library (Moon et al., 1985) with NF70 antibodies. Preliminary genomic DNA blot analysis revealed that these NF70 cDNAs derived from mRNA coded from a gene that is represented similarly as single copy in the haploid chicken genome. On the other hand, RNA blot analysis revealed the hybridization of this cDNA to a single RNA band of about 2.4 kb in retina, spinal cord, cerebellum and whole brain RNA. Similar mRNA blots with erythrocyte RNA reveals also a single mRNA species of slightly lower molecular weight. Positive signals obtained with skeletal muscle RNA derive most possibly from cross-reaction with desmin. Further studies regarding the confirmation of the identity of these clones as well as the structure and expression of their genes are under way.

References:
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184. Synthesis and Assembly of Erythrocyte Band 3

John V. Cox

Previous studies have shown that anion erythrocyte band 3 is composed of two antigenically and structurally similar polypeptides of 100 kd and 105 kd (Cox et al., 1985). To investigate the synthesis and assembly of these two polypeptides, pulse-chase studies have been performed. Immunoprecipitates from pulse-labeled erythrocytes that have been hypotonically lysed indicate that the band 3 polypeptides are rapidly inserted into the endoplasmic reticulum. Digestion of these immunoprecipitates with endoglycosidase H (endo H) prior to gel analysis has revealed that band 3 is synthesized as 95 kd and 100 kd precursors, each of which undergoes endo H-sensitive glycosylation. Further digestion studies of intact cells with proteinase K conjugated to cellulose beads has shown that all of the newly synthesized band 3 is rapidly inserted into the endoplasmic reticulum. Digestion of these immunoprecipitates with endoglycosidase H (endo H) prior to gel analysis has revealed that band 3 is synthesized as 95 kd and 100 kd precursors, each of which undergoes endo H-sensitive glycosylation. Further digestion studies of intact cells with proteinase K conjugated to cellulose beads has shown that all of the newly synthesized band 3 is rapidly inserted into the endoplasmic reticulum. Digestion of these immunoprecipitates with endoglycosidase H (endo H) prior to gel analysis has revealed that band 3 is synthesized as 95 kd and 100 kd precursors, each of which undergoes endo H-sensitive glycosylation. Further digestion studies of intact cells with proteinase K conjugated to cellulose beads has shown that all of the newly synthesized band 3 is rapidly inserted into the endoplasmic reticulum.
the polypeptides undergo additional modifications resulting in further molecular weight shifts. These polypeptides also undergo a transition, over a period of hours, from a detergent-soluble to a detergent-insoluble form, possibly by binding to the membrane cytoskeleton. The remainder of the band 3 molecules are internalized in a vesicular compartment, the peak of internalization occurring two hours after the initiation of synthesis. The fate of these internalized molecules is at this time unknown. Preliminary experiments have also suggested that as erythropoiesis proceeds from day 10 to day 15, the conversion of band 3 from a detergent-soluble to a detergent-insoluble form decreases, thereby providing a potential mechanism for regulating the assembly of the membrane cytoskeleton during development.

In conjunction with these studies at the protein level, attempts are currently being made to S1 map the erythroid band 3 transcripts through the use of RNA probes. Previous Northern analysis has revealed a single band 3 mRNA of 4.4 kb (Cox et al., 1985). It is hoped that the relationship between the 95 kd and 100 kd band 3 precursors and this 4.4 kb mRNA species will be clarified by these studies.

References:

185. Isolation and Characterization of the Gene Coding for the Chicken Erythrocyte Anion Transporter

Yassemi G. Capetanaki, Lois M. Banta

cDNA probes specific for the chicken erythrocyte anion transporter have been isolated recently (Cox et al., 1985) and used to show that this protein is most likely coded by a single gene. This gene is transcribed into at least two distinct mRNAs that show cell specificity in their expression. A 4.4-kb-long mRNA accumulates in chicken erythrocytes, whereas in kidney cells a slightly lower molecular weight RNA (4.3 kb) is expressed, which codes for a polypeptide distinct in size from its erythrocyte counterparts (see Abstract No. 184). Since the chicken erythrocyte anion transporter is composed of at least two polypeptides (Jay, 1983; Cox et al., 1985), it might be possible that there is more than one anion transporter mRNA in the erythrocytes with very similar electrophoretic mobilities. Hence, several mechanisms may be involved in generating the RNA and protein diversity in the same or different cell types. A complete answer to this question would require the isolation and characterization of the gene and its transcripts. Such studies will further confirm the existence of a single chicken anion transporter gene. Furthermore, in vitro mutagenesis and subsequent transformation of this gene would yield much information regarding the different functional domains of the anion transporter, as well as the domains responsible for correct modification, segregation and assembly of this important transmembrane protein. For these purposes we have attempted to isolate and characterize this gene. We have screened two different chicken genomic libraries with the anion transporter-specific cDNA probe. The first library is a chicken Charon 4A prepared by partial digestion with HaeIII and AluI (a gift from Dr. B. Dodgson). The second one was prepared by partial Mbol digestion and insertion into the BamHI polylinker site of the EMBL-4 vector (a gift from Dr. C. Hodgson). Several clones have been isolated from both libraries and are currently being characterized.

References:

186. Anion Transporter (Band 3) in Muscle Cells: Transient Expression during Myogenesis

Lois M. Banta, John V. Cox

Recent studies from this laboratory have demonstrated the presence of a protein that is related to the erythrocyte anion transporter (band 3) in adult chicken kidney cells (Cox et al., 1985). To investigate the expression of band 3 during chicken embryogenesis, frozen sections of embryos were examined by indirect immunofluorescence, using affinity-purified antibodies against chicken erythrocyte band 3. Employing this technique, we have observed the transient appearance in skeletal and cardiac muscle cells of a molecule that cross-reacts with the band 3 antibody. Expression of this band 3-related antigen is limited to the period during embryogenesis corresponding to the time of muscle cell fusion (in the case of skeletal muscle). The antigen in vivo is localized along the muscle cell membrane prior to the appearance of striations, but is excluded from heavily striated areas of the myotube.
In order to study in more detail the expression of this polypeptide during myogenesis, we have used in vitro myocultures. Examination of cultured myotubes by immunofluorescence and by immunoblotting reveals a transient appearance of this antigen similar to that seen in vivo: band 3 appears at the onset of cell fusion, reaches its peak of expression by the time most of the cells are multinucleate, and disappears as the cells become heavily striated. The observed fluorescence appears to be localized in adhesion plaques, similar to those seen for vinculin, and is concentrated at the ends of the myotubes, suggesting a role for band 3 in cell-substratum attachment and possibly in cell fusion. Immunoblotting of membrane fractions from myotubes in vitro and from muscle cells in vivo reveals a single cross-reacting polypeptide of molecular weight slightly higher than that of the erythrocyte band 3. Preliminary immunoprecipitates obtained with this antibody from cultured myotubes labeled with $^{[35S]}$methionine or with $^{[3H]}$DIDS (a ligand specific for band 3) also contain a species of similar molecular weight. Current experiments are being conducted to determine the extent of association of this polypeptide with the cytoskeleton and its possible co-localization with other adhesion molecules. Other experiments are in progress to demonstrate the presence of a band 3-related mRNA species in muscle by hybridization with band 3 cDNA clones isolated in this laboratory. Future studies will attempt to elucidate the role of band 3 in muscle cell fusion and attachment to the substratum.

References:

187. Fatty Acid Acylation of Erythroid Ankyrin

Matthias Staufenbiel

Ankyrin is an extrinsic membrane protein in vertebrate erythrocytes. It functions to link spectrin to an intrinsic membrane protein, the anion transporter. Ankyrin thereby serves to anchor the peripheral (membrane) cytoskeleton to the plasma membrane bilayer. We found that ankyrin is one of the most heavily labeled proteins when erythroid cells are incubated with $^{[3H]}$palmitic acid. A labeling due to conversion of the fatty acid into amino acids and their incorporation into the polypeptide backbone can be excluded since the pattern of proteins labeled with palmitic acid is strikingly different from the pattern obtained after labeling of the cells with amino acids. The specificity of the labeling is also demonstrated by the fact that $^{[3H]}$myristic acid is not incorporated into ankyrin. The fatty acid associated with ankyrin is not removed by boiling in SDS and subsequent gel electrophoresis or by chloroform/methanol extraction. However, the palmitic acid label can be released largely from ankyrin by treatment with hydroxylamine. Taken together, this supports the conclusion that the fatty acid is covalently bound to the polypeptide chain. Previous studies of fatty acid acylated proteins have identified (parts of) the endoplasmic reticulum or the Golgi apparatus as the site of acylation. Since ankyrin after its synthesis at "free" polysomes is rapidly assembled onto the membrane cytoskeleton, it appears likely that it is fatty acid acylated via a different mechanism. Acylation can occur even after assembly at the plasma membrane because palmitic acid labeling of ankyrin continues after inhibition of protein synthesis, conditions under which only membrane-associated ankyrin is present. These results suggest that fatty acid acylation of ankyrin serves to modify the function of this protein after its assembly onto the membrane cytoskeleton. Attachment of fatty acids to ankyrin may provide a mechanism for lipid organization in the bilayer of the red blood cell.

188. Fate of the Excess Unassembled Spectrin Subunits during the Biogenesis of the Spectrin-Actin-based Membrane Skeleton in Erythroid Cells

Catherine M. Woods

Red blood cells contain a characteristic membrane skeleton that is responsible for maintaining cell shape and conferring tensile strength to the plasma membrane. The principle component of this skeleton is the protein spectrin, which consists of an $\alpha$-spectrin ($M_r = 240,000$) and $\beta$-spectrin ($M_r = 220,000$) heterodimer. Spectrin cross-links short actin filaments to form an anastomising network underlying the plasma membrane. This network is linked to the plasma membrane through the binding of the $\beta$-spectrin subunit to ankyrin which in turn binds to the transmembrane anion transporter (reviewed by Branton et al., 1981).

Previous studies from this laboratory have revealed that throughout the development of the chicken
erythrocyte, the peripheral components of the erythroid membrane skeleton—ankyrin, α-spectrin and β-spectrin—are synthesized in a soluble form in the cytoplasm in non-stoichiometric amounts that are in excess of the amounts that assemble onto the membrane skeleton (Blikstad et al., 1984; Moon and Lazarides, 1984). These results clearly indicate that stoichiometry and topology of the membrane skeleton are determined by posttranslational mechanisms. One important regulatory mechanism controlling topology appears to be an inherent instability of the un­assembled polypeptides that become stabilized against intracellular proteolysis upon binding to the membrane skeleton. Our studies have revealed that the degradation of the excess unassembled spectrin subunits is brought about by separate pathways in a highly selective fashion. Excess soluble β-spectrin is turned over extremely rapidly with a \( t_{1/2} \) of 15-20 min by a neutral cytoplasmic protease system. In contrast, excess α-spectrin is turned over more slowly (\( t_{1/2} = 2 \) hr) and is degraded by a pathway with all the pharmacological characteristics of a lysosomal-type pathway. Unassembled ankyrin exhibits an intermediate half-life (\( t_{1/2} = 1 \) hr) and appears to be degraded in the cytoplasm. The extremely selective and rapid degradation of soluble unassembled β-spectrin has prompted us to suggest that this apparently wasteful process of degrading a protein as soon as it is synthesized might serve to ensure correct topology of the spectrin-actin network by preventing anomalous interactions between the peripheral components from occurring within the cytoplasm (Woods and Lazarides, 1985).

The available evidence suggests that newly-synthesized ankyrin, α- and β-spectrin polypeptides are only competent to assemble onto the membrane skeleton for a short time after synthesis. Our recent studies have focused on analyzing the nature of these unassembled soluble pools of the spectrin subunits. We have discovered that unassembled soluble α- and β-spectrin exist in the form of homo-oligomers. These homo-oligomers are incompetent to interact with each other or with ankyrin. However, if these homo-oligomers are mixed, denatured and allowed to re-­nature, the predominant form that is formed is the αβ-heterodimer. Thus, these homo-oligomeric forms of spectrin represent alternative conformations that the newly-synthesized monomers can adopt to the heterodimeric form found in the membrane skeleton. Homo-oligomerization may represent the mechanism by which α- and β-spectrin are targeted for separate pathways of degradation. In addition, it clearly provides an additional mechanism to prevent interactions between the excess unassembled subunits.

References:

189. Assembly of Protein 4.1 Onto the Membrane Cytoskeleton during Erythropoiesis
Matthias Staufenbiel
A network of cytoskeletal proteins is attached to the cytoplasmic surface of the vertebrate erythrocyte plasma membrane. This network is thought to contribute to the shape, deformability, and elasticity of the cell. It is based on actin oligomers bound and cross-linked by spectrin tetramers. Protein 4.1 strongly enhances the interaction of spectrin and actin by forming a stable ternary complex with these proteins and thus can serve to strengthen the network. Protein 4.1 also may link the actin-spectrin network to the membrane bilayer in addition to the well characterized ankyrin-mediated anchorage of spectrin to the anion transporter. Chicken erythrocyte protein 4.1 was found to exist as a family of related polypeptides (Granger and Lazarides, 1984). It was shown that the pattern of the protein 4.1 polypeptides changes during terminal differentiation of erythroid cells at a time when the cells become postmitotic (Granger and Lazarides, 1985). To further understand the significance of this switch between protein 4.1 polypeptides expressed, we have determined their amount at the membrane cytoskeleton. The quantity of protein 4.1 in erythroid cells derived from embryos of different ages was measured relative to spectrin, the cytoskeletal component with which protein 4.1 interacts. The protein 4.1 variants of premitotic erythroid cells are present at the membrane cytoskeleton in considerably smaller molar amounts than spectrin. However, later in erythropoiesis (postmitotic cells), an equimolar ratio of protein 4.1 and spectrin is
observed. This increase in the protein 4.1:spectrin ratio occurs in both the primitive and the definitive erythroid cell series. It is not a cell lineage-specific phenomenon, but is related to erythroid terminal differentiation. The increased amount of protein 4.1 at the final stages of erythropoiesis and the ability of protein 4.1 to strengthen the membrane cytoskeleton suggests that the protein 4.1 accumulation may serve as a mechanism leading to the final stabilization of the erythrocyte membrane cytoskeleton. To better understand this process we have studied the assembly of protein 4.1 onto the membrane cytoskeleton during chicken erythrocyte development. At all stages of erythropoiesis the assembly of protein 4.1 is extremely rapid with a half-life of less than 1 min for a newly synthesized molecule to be assembled. Assembly is highly efficient since only a very minor fraction of the newly synthesized molecules is not assembled immediately. The latter fraction has a substantially longer half-life (about 20 min) and presumably is degraded. Cotranslationally or immediately after termination, newly synthesized protein 4.1 molecules must be allocated to either of the two subpopulations and then go through different pathways in the cell. The ratio of the different protein 4.1 polypeptides, however, is not altered during this process, during assembly, or thereafter at the cytoskeleton. We conclude that the amount and the variant ratio of protein 4.1 assembled are determined largely at the transcriptional and/or at the translational level.

References:

190. Isolation of cDNA Clones for Chicken Protein 4.1 and Analysis of Protein 4.1 mRNA

John J. Ngai, Randall T. Moon

A protein meshwork based on the proteins spectrin and actin lines the cytoplasmic surface of the mammalian erythrocyte, and is believed to confer upon this cell's membrane its properties of strength and elasticity. Protein 4.1 facilitates the interaction of spectrin and actin by binding to both of these components and may also interact directly with the plasma membrane. Thus, protein 4.1 plays a key role in the maintenance of the membrane cytoskeleton. In chicken erythrocytes, protein 4.1 exists as six major variants of 87, 100, 115, 150, 160 and 175 kilodaltons (kd), with the 100 kd and 115 kd variants in greatest abundance (Granger and Lazarides, 1984). However, in immature, mitotic chicken erythroblasts, the 87 kd polypeptide predominates, and as the cells mature, the 100 kd and 115 kd polypeptides are found at higher levels (Granger and Lazarides, 1985). Chicken lens cells also express protein 4.1; in mitotic lens epithelium, the 87 kd and 150 kd variants predominate, with minor amounts of the 175 kd and 77 kd polypeptides present, whereas in the mature postmitotic lens fiber, the 175 kd and 160 kd protein 4.1 variants are expressed almost exclusively. Hence, the expression of protein 4.1 polypeptides in chickens displays both tissue-specific and developmentally-regulated patterns of expression.

In order to define the mechanism(s) by which protein 4.1 variants are generated, we have isolated cDNA clones encoding protein 4.1 from a chicken erythroid cDNA λgt11 expression library, using antiprotein 4.1 antiserum as a probe. The identities of the cDNAs were confirmed by positive hybrid-selected translation. RNA blot analysis demonstrates the existence of a single-size protein 4.1 RNA of 6.5 kb in lens as well as in erythroid cells from 4-, 10-, and 15-day-old embryos. Multiple variants of protein 4.1 therefore appear to arise from a single size class of mRNA. To our surprise, in vitro translation of erythroid RNA in a rabbit reticulocyte lysate yields a protein 4.1 pattern quantitatively different than the pattern observed in vivo: the 150-175 kd variants are the major polypeptides synthesized in vitro, with lower quantities of the 87-115 kd variants present. To determine if all the protein 4.1 variants are encoded by the 6.5 kb RNA, we separated erythroid poly(A)+ RNA on a preparative methyl mercury hydroxide gel and used the RNA from each gel slice for in vitro translation and RNA blot analysis. The results show that all protein 4.1 polypeptides are synthesized in vitro from each RNA fraction that co-migrates with the 6.5 kb protein 4.1 mRNA. How do multiple protein 4.1 polypeptides with stage- and tissue-specific variations arise from a single RNA? Cell-specific co-translational or limited posttranslational processing of a parent protein 4.1 polypeptide may occur. Alternatively, or additionally, multiple protein 4.1
mRNAs with similar or identical sizes may be expressed. Genomic DNA blotting reveals the presence of a single protein 4.1 gene in chickens. Therefore, putatively multiple protein 4.1 mRNAs may be generated by regulated, alternative processing of primary protein 4.1 RNA transcripts. We are currently testing for the presence of multiple protein 4.1 mRNAs by nuclease-protection transcriptional mapping experiments.

References:

PUBLICATIONS

Professor: Jean-Paul Revel
Senior Research Associate: S. Barbara Yancey
Visiting Associates: Rolf Dermietzel, Birgit Satir
Graduate Student: Jan H. Hoh
Research Staff: Janice M. Cline, Jean E. Edens, Patrick F. Koen

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Summary: My lab has continued working on characterization of the gap junction protein. We have succeeded in preparing an antibody against the liver 28 kD component, and have affinity purified it. It does not cross-react with either heart junction protein or with the lens main intrinsic protein (MIP), believed to represent the junction protein in the lens. We had hoped for some cross-reactivity since there is a 43% match of residues at the amino end (the only part sequenced) of heart and liver. We also have worked on cloning the liver gap junction protein, one attempt being made by Jan Hoh and the other in collaboration with N. Davidson, B. Yancey and B. Nicholson working together.
With a visitor from Germany (Rolf Dermietzel) we devised a methodology for the simultaneous detection of junctions between cells by immunocytochemistry and of the position of the same cells in the mitotic cycle using a monoclonal antibody against BrdU incorporated into DNA. In regenerating liver we can now follow cell proliferation and enumerate junctions at the same time. We have learned that junctions disappear during the S phase and not as had been believed, at the peak of mitosis.

Based on our recently published sequence for MIP we have proposed a model for the arrangement of this molecule in a junction. Peptides have been synthesized corresponding to two regions of the molecule, one theoretically exposed at the cytoplasmic face, the other predicted to be buried in the intercellular space, between the cells where it should be inaccessible to enzymes. Immunoblots of controls, trypsin-treated and chymotrypsin-treated lens membranes, support our predictions. Further work along these lines is in progress.

Besides Dermietzel, another visitor, Birgit Satir from Albert Einstein College of Medicine, spent six months in the lab.

191. Isolation of a Gene Encoding the Main Intrinsic Protein (MIP) of the Rat Lens

S. Barbara Yancey, Janice M. Cline, Jean-Paul Revel

Previous work in our laboratory (Biology 1983, Abstract No. 137; 1984, No. 161) has led us to believe that gap junction proteins from different tissues are encoded by different, more or less related genes which we hope to characterize. As a first step, we have used our cDNA clone for the main intrinsic protein from bovine lens (a putative gap junction protein; Biology 1984, Abstract No. 159) to screen a rat genomic library constructed in Charon 4A (Sargent et al., 1979). Six positive clones were selected after rescreening. Hybridization of 5' and 3' cDNA probes to blots of cloned DNA digested with EcoRI indicates that one of the genomic clones has an insert of about 10 kb, which contains both 5' and 3' sequences encoding the rat MIP. We have also identified two clones with inserts that encode only 5' or 3' sequences. We have begun mapping the gene by analysis of restriction endonuclease digests of the three clones and rat genomic DNA after hybridization with nick-translated cDNA probes representing either 5', middle, or 3' sequences.

References


192. Construction of Sense and Antisense mRNA Probes for Study of the Developmental and in vitro Expression of MIP

S. Barbara Yancey, Janice M. Cline, Jean-Paul Revel

In order to study the expression of MIP during lens development and in vitro in cells in which it is not normally present (e.g., Xenopus oocytes), we have re-assembled our two overlapping cDNA clones (Biologie 1984, Abstract No. 159) in the SP6 vector (Promega Biotic) in constructions that allow us to transcribe full length sense and antisense mRNA.

Preliminary to experiments on the in vitro expression of MIP, we are presently investigating the feasibility of using Xenopus oocytes as a system for microinjection of capped MIP mRNA transcribed from the SP6 clone (Konarska et al., 1984). The appearance of functional membrane channels between paired oocytes would be determined from the cell-to-cell transfer of fluorescent dye. The use of cell lines deficient in cell-cell communication will also be explored.

References


193. Orientation of MIP in Lens Membranes

Jan H. Hoh, S. Barbara Yancey, Janice M. Cline, Jean-Paul Revel

The 26 kd main intrinsic protein (MIP26) of the lens is thought to be the major protein component of the lens gap junction. The recent isolation of a cDNA coding for MIP26 has revealed the complete amino acid sequence of the protein (Revel and Yancey, 1985). From analysis of the sequence, a model for the molecular organization of MIP26 has been proposed. To test this model and later probe the function of MIP26, we have produced antisera against two commercially synthesized peptides. According to the model, the first of these peptides (LP1) is located at the C-terminal end of the protein near its exit from the membrane. The second peptide (LP2) is located on the extracellular side in the "gap" between two transmembrane helices.
Site-directed antibodies were used in blotting experiments to determine the sidedness of MIP. Bovine lens membranes were digested respectively with trypsin and chymotrypsin. If the model proposed was correct, we expected that the most C-terminal peptide (LP1) would be removed, but that the other (LP2) would not be affected by the enzyme treatment. This in fact proved to be the case. These data support the idea that MIP is a junctional molecule, and suggest that the model deduced might be correct at least in its general features.

Reference:

194. Differentiation of the Lens
S. Barbara Yancey, Jeanne Weaver¹, Janice M. Cline, Jean E. Edens

The lens is an unusual tissue in that it is comprised originally of a single cell type. The cells of the anterior epithelium continuously differentiate into fiber cells, which form the bulk of the lens. Thus, the complete pathway of cell differentiation at all stages can be viewed in a single lens. During differentiation there appears to be a turn off of the expression of one type of gap junction and the turn on of expression of a gap junction that is different in morphology as well as biochemical and physiological characteristics. MIP in oligomeric form is believed to form the hydrophilic channels of which this second type of junction is comprised. Since the epithelial cells are coupled not only to each other but also to differentiated lens fiber cells, it is possible that the epithelial cells are capable of expressing two different types of gap junctions. To address this question, we have prepared sections of rat lens by a modification of the technique of Cox et al. (1984) and will use our antisense probe for in situ hybridization to determine the distribution of MIP mRNA in the different regions of the lens.

Reference:

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195. Antibodies to the Rat Hepatic Gap Junction Protein
Jan H. Hoh, Jean-Paul Revel

Antiserum against the 28 kd protein component of the rat hepatic gap junction was produced by immunizing rabbits with SDS-solubilized isolated gap junctions. This antiserum was affinity purified against SDS-solubilized gap junctions coupled to CNBr-Sepharose. The antiserum does not cross-react with isolated heart gap junctions, nor with the two 10 kd fragments generated by treating gap junction plaques with trypsin. The latter result suggests that the antiserum only recognizes cytoplasmic determinants of the gap junction since the 10 kd fragments are thought to be regions of the molecule protected by the membrane. We are currently using this antiserum for immunocytochemical identification of gap junctions and molecular cloning using the expression vector λgt11.

196. λgt11 Cloning of the Rat Hepatic Gap Junction Protein
Jan H. Hoh, Jean-Paul Revel

Using a polyclonal antibody against the 28 kd component of the rat hepatic gap junction, we have screened a rat liver and a human hepatoma λgt11 library. Initially, we isolated a 2.2 kb clone from the rat liver library using the whole serum. This clone was subsequently found to react with a contaminating activity in antiserum, presumably against a highly antigenic contaminant of the gap junction preparation used for immunization. The antiserum was then affinity purified to remove this activity. Using the affinity-purified antibody, we have now isolated a clone from the hepatoma library with a 130 bp insert. While this is quite small, it is easily large enough to code for an antigenic site. Since the chances are small that this cDNA contains any of the known protein sequence that would allow us to positively identify it, we intend to use it as a probe to isolate the complete cDNA for the hepatic gap junction protein.

197. In Search of the Liver Gap Junction Gene
Bruce J. Nicholson¹, S. Barbara Yancey, Norman D. Davidson¹, Jean-Paul Revel

In order to further our structural and comparative studies on the gap junction protein, we have been
interested for some time in isolating a cDNA clone encoding the rat liver gap junction protein. Several attempts using various synthetic oligonucleotides deduced from the known amino-terminal protein sequence as direct probes of Northern blots and cDNA libraries in both λgt10 and pBR322 have failed to identify the mRNA or its cDNA (Biology 1982, Abstract No. 131; 1983, No. 140; 1984, No. 162). Likely reasons for this lack of success include the poor kinetics and low signal-to-noise ratio inherent in oligonucleotide probes and the possible underrepresentation of sequences encoding the NH₂ terminus in a cDNA library. Currently, a new approach is being used to circumvent at least these major difficulties.

While the oligonucleotide probes have failed to give a signal in filter hybridizations, they have consistently worked as primers of reverse transcriptase using rat liver poly(A)+ RNA as a template. An oligonucleotide representing the complementary strand to that encoding amino acids 15-24 (degenerate positions guessed based on the frequency of codon usage) uniquely primes the synthesis of a 140 base cDNA. This in turn is recognized by the oligonucleotide encoding amino acids 1-14. The yield of this cDNA, which appears to contain the first 72 bases of coding sequence along with about 68 bases of 5' untranslated region, would suggest that it is derived from a very low abundance message. In order to obtain sufficient amounts of material for screening, we are currently tailing this cDNA (after elution from a gel) for cloning into a pEMBL plasmid. This flexible vector system combines the advantages of plasmid chemistry with a dideoxynucleotide sequencing system. Once cloned, single-stranded or nick-translated probes of this cDNA will be used for screening the genomic library of Sargent, thus avoiding any bias against 5' sequences of the mRNA.

Genomic clones selected can be verified by direct sequencing and comparison with the protein sequence established earlier (Biology 1980, Abstract No. 136). The complete protein sequence can then be deduced from cDNA clones identified using a portion of the genomic clone to screen the rat liver λgt10 library constructed previously (Biology 1984, Abstract No. 162). These cDNA and genomic clones will also initially be used to address questions relating to processing of the junctional protein and the extent and diversity of what appears to be a family of junctional genes.

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198. Is There a Relationship between Mitosis and Gap Junctions?

Rolf Dermietzel, S. Barbara Yancey, Jean-Paul Revel

In previous work we have shown that at a specific time during liver regeneration, there is very sharp drop in the number of gap junctions found between hepatocytes, accompanied by characteristic changes in cell-cell coupling. Although the time of junction loss seemed to be associated with the first wave of mitosis, it was technically difficult to clearly correlate junction loss with a specific time in the cell cycle (Yee and Revel 1978; Meyer et al., 1981). Divisions do not take place synchronously in the whole liver, and freeze fracture, until recently the best way of assessing the number of gap junctions, does not permit one to judge the mitotic stage. Recently, a monoclonal antibody that binds specifically to BrdU even after incorporation into DNA (Gratzner, 1982) has become commercially available. We have used this reagent to mark DNA synthesis just as 3H-thymidine is used for autoradiography. In combination with immunostaining for gap junction protein, this allows one to examine the same cell for presence or absence of junctions and its position relative to the S phase. We find that the junction loss actually begins during DNA synthesis, rather than being associated with mitosis itself. We are presently pursuing the kinetics of cell division and correlating these data with the cycle of disappearance and resynthesis of gap junctions.

References:

199. Immunocytochemistry of Gap Junction Formation and Disappearance

Rolf Dermietzel, Jean E. Edens, S. Barbara Yancey, Jean-Paul Revel

The preparation of antibodies against gap junction protein (see Abstract No. 195) permits the identification of gap junction plaques at the ultrastructural level using an indirect technique where a second anti-
body is labeled with gold particles or ferritin. After cryoultramicrotomy of sections frozen in sucrose, it becomes possible to identify intracellular stores of gap junction protein, as well as membrane-associated protein. In normal liver most of the gold particles denoting the presence of the antigen are found associated with morphologically recognizable gap junctions. Too few gold particles are found elsewhere in the cell to suggest the presence of gap junction protein in other structures or organelles. After heptectomy, however, at times when one expects the reformation of junctions after their eclipse, small cytoplasmic vesicles are found deep in the cell or close to gap junctions. The label seems to be associated with the lumen of these vesicles, not with their membranes. One might have predicted a membrane location for protein destined to play a role in junction formation. Because the vesicles are seen mainly at times when junction formation should take place, and not at times when junctions disappear from the cell surface, we tend to believe that these vesicles represent the vehicle for insertion of new protein into the membranes, rather than uptake of old, spent protein. The mechanism of junction removal is still mysterious. They seem to disappear in a way that involves not only the loss of cell contact and of membrane particles recognizable by freeze cleaving, but also in a way that causes a loss of immunogenicity.

**PUBLICATIONS**


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**Assistant Professor:** Ellen Rothenberg

**Research Fellows:** Barry I. Caplan, Christine Kinnon, James P. Lugo, Kathleen L. McGuire

**Graduate Student:** Thomas J. Novak

**Associate Biologist:** Rochelle A. Diamond (Sailor)

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**Summary:** Our laboratory is interested in the sequence of developmental events by which hematopoietic precursor cells become T (thymus-derived) lymphocytes. Mature T lymphocytes regulate all immune responses and carry out target antigen-specific killing. They recognize (bind and discriminate) specific foreign antigenic structures, secrete a unique set of polypeptide hormones in response to antigen binding, and proliferate when activated by antigen binding. These physiological properties depend on the activation of an increasingly well-defined set of genes. Genes encoding components of the T-cell receptor are expressed constitutively, while genes encoding the hormones, growth factors, and growth-factor receptors are inducible. Both the expression of the first set of genes and the inducible state of the second set of genes are specific to the T cell lineage and must be acquired as a function of differentiation. We are therefore tracing these changes in gene activity and cell physiology as landmarks in T-lymphocyte development.

T cells acquire most of their distinctive characteristics in the thymus gland, an organ that serves as a
way station for T-lineage precursors between the bone
marrow, where they originate, and the peripheral
lymphoid organs, such as the spleen and lymph nodes.
The thymus is therefore a rich source of cells in many
intermediate stages of development. Over the past
several years, we have learned to resolve and isolate
these various populations, and have begun to trace
their precursor-product relationships. An early pre­
cursor cell class, called "double negative" (dbl neg) for
its lack of two T cell-specific marker proteins, gives
rise to two general types of differentiated progeny.
The first type comprises mature T cells of various
functional subclasses, such as helper, killer, and
suppressor T cells. The second and more enigmatic
type consists of functionally inert cells expressing a
unique pattern of T-cell markers, which accumulate in
the cortex of the thymus and then die. We are studying
the sequence of events that result in maturation, and
in particular, trying to define the choice points where
the maturation and death lineages diverge.

In the past year, we have focused on two gene
products crucial for mature T cell function: the T cell
growth factor interleukin-2 (IL-2) and the receptor for
interleukin-2 (IL-2R). These products control all the
proliferative behavior of mature T lymphocytes. They
provide the link between antigen recognition and
growth because both genes are expressed only when
the cell is triggered by antigen. The ontogeny of IL-2
and IL-2R expression is thus related to the develop­
ment of the antigen receptor signaling mechanism, as
well as to the changes in intrinsic chromatin structure
that may render the IL-2 and IL-2R genes inducible. In
the past year, considering one of these genes, we have
been able to dissect this process into several distinct
stages: cells in which the IL-2 gene is not yet
inducible; cells in which the IL-2 gene is inducible, but
the antigen-binding receptor is not yet capable of
triggering induction; and cells in which the IL-2 gene is
induced when the antigen receptor is engaged. Our
data show that the IL-2 and IL-2R genes are activated
independently in development. We are still inves­
tigating their roles in these early stages, for we find
that most proliferation in the thymus must be driven
by an IL-2-independent mechanism, in sharp contrast
to the case of mature cells. Through collaboration
with Prof. Lee Hood's group, we have obtained probes
for the mRNAs of the different antigen receptor
chains, enabling us to relate these changes in respon­
siveness to changes in possible receptor structure.

This mosaic of observations can serve as the basis
for a rigorous developmental analysis if it is possible
to isolate some of these processes for study in vitro.
Some very recent results indicate that we will in fact
be able to do so. By activation of double negative
precursor cells with signals that bypass the antige­
binding receptor, we stimulate them to grow in vitro.
Preliminary evidence suggests that they may retain
their precursor activity under these growth conditions.
If these indications are borne out, we may not only be
able to isolate T-cell precursors for study of their
cloned descendants, but also make them susceptible to
manipulation by gene transfer.

We have reached the point where this application is
not only attractive but also feasible. Our research has
established conditions for introduction of exogenous
DNAs into T cells, and explored several parameters for
controlling their levels of expression. In the next few
years, we plan to apply these protocols to genes impor­
tant in growth control, such as the IL-2 gene. These
experiments should ultimately lead from initial testing
of response perturbations in cloned T-cell lines to
engineering of developmental perturbations in thymic
precursor cells.

200. Proliferation of Intrathymic Stem Cells With and
Without Interleukin-2 Receptors

James P. Lugo, Santosh N. Krishnan¹,
Rochelle A. Diamond, Ellen Rothenberg

Mature T cells invariably express interleukin-2
receptors (IL-2R) during antigen-driven proliferation
(Cantrell and Smith, 1983, 1984). We have shown that
most (90-95%) proliferating thymocytes, by contrast,
do not express IL-2R (Lugo et al., 1985). Two
monoclonal antibodies (7D4 and 3C7) specific for the
mouse IL-2R (Ortega et al., 1984) were used in these
studies. Thymocytes were separated into dividing and
postmitotic populations on the basis of size, and
further subdivided on the basis of binding to the lectin
peanut agglutinin (PNA). PNA-binding cells (PNA⁺)
represent most or all of the committed cells in the
major cortical lineage, most of which will die, while
PNA⁻ cells include both mature cells and some early
precursors. Cells expressing and synthesizing IL-2R
were found almost exclusively in PNA⁻ populations.
Nevertheless, only a minority (10-15%) of the PNA- blast population expressed this receptor. PNA+ (cortical) blasts contained few (0.1-1.0%) cells that were IL-2R positive. More recently, antibody plus complement cell lysis studies have confirmed these results.

Other recent studies (Ceredig et al., 1985; Raulet, 1985) have indicated that IL-2R+ thymocytes can be found in the highly immature Lyt-2-, L3T4- (double negative; dbl neg) cell class. We have confirmed and extended these results using fluorescence cytometry and complement-mediated cell lysis. Both approaches yielded similar results: of the 1-2% of unfractionated adult thymocytes expressing IL-2R, over 90% must be Lyt-2-,L3T4+. Direct isolation and phenotypic characterization of dbl neg thymocytes revealed that about 45-55% of these cells expressed IL-2R, suggesting that dbl neg thymocytes are themselves developmentally heterogeneous.

Overall, these findings are paradoxical, for they confine IL-2R+ thymic lymphoblasts to an immature T-cell population, while most of the intrathymic blast cells (both cortical and medullary) are dividing in response to signals that are independent of IL-2 (Lugo et al., 1985). This conundrum is accentuated by two additional observations. First, dbl neg thymocytes are not among the most actively proliferating cells in the thymus, based on their DNA content, and, second, these cells express only a low affinity form of the IL-2R. Unlike mature T cells that express IL-2R, thymic lymphoblasts do not grow in vitro in the presence of high levels of IL-2. These data are difficult to reconcile in the context of current thymus models, most of which suggest that antigen initiates both thymic differentiation and division. For mature T cells, antigen binding invariably leads to IL-2R expression. The failure of otherwise mature thymic lymphoblasts to express this receptor implies that antigen may not be the initial signal that triggers pre-T-cell mitogenesis. If antigen, including self-histocompatibility antigen, plays any role, it may act instead as a developmental signal, possibly controlling lineage commitment.

References:

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201. Transient Appearance of Thymocytes with Low Numbers of Interleukin-2 Receptors

James P. Lugo, Ellen Rothenberg

We have extended our phenotypic analysis of IL-2R+ cells to include fetal and neonatal thymocytes. By fluorescence cytometry, we find that 3-8% of neonatal thymocytes express IL-2R. However, by complement elimination, 50-60% of these cells express this receptor. In addition, stimulation of neonatal thymocytes and their IL-2R- counterparts revealed that the IL-2R- cells produced 4-5 fold more IL-2 than expected even if all the IL-2 producers were IL-2R+.

These observations have led us to conclude that many neonatal thymocytes, presumably of a mature cortical or medullary phenotype, express higher affinity IL-2R below our fluorescence cytometric level of detection. The high-affinity receptors on these cells could absorb IL-2, artifactually reducing the amount of IL-2 apparently produced by the total neonatal population. This would provide the only example of IL-2R+ thymocytes that were not of the dbl neg class. We are currently testing this hypothesis using radiolabeled IL-2 binding. Should neonatal thymocytes express this form of the IL-2R, it may suggest a role for IL-2-driven intrathymic proliferation in early life that does not appear to exist once the mouse reaches young adulthood.

202. Interleukin-2 Production by Lyt-2-,L3T4- Thymocytes

James P. Lugo, Santosh N. Krishnan, Ellen Rothenberg

Increases in intracellular calcium and activation of protein kinase C play important roles in T-cell activation and mitogenesis (Imboden and Stobo, 1985; Imboden et al., 1985). This fact prompted us to stimulate highly immature T cells with the calcium ionophore A23187 and 12-o-tetradecanoyl phorbol 13-acetate (TPA) in order to determine whether pre-T cells acquire the ability to produce IL-2 before they
acquire the ability to respond to antigen. Immature T-cell populations were obtained by eliminating thymocytes that expressed the Lyt-2 and L3T4 cell-surface proteins. These remaining "double negative" (dbl neg) cells were stimulated for IL-2 production using Concanavalin A (ConA) or the calcium ionophore A23187, together with TPA. Using this approach, we found that dbl neg thymocytes produce IL-2 only when stimulated with A23187 + TPA. When tested on more mature T-cell populations, by contrast, both ConA and A23187 were potent inducers of IL-2 synthesis, with ConA consistently eliciting 20-25% as much IL-2 per cell as did A23187. Additional experiments ruled out kinetic and dosage effects as possible explanations for why ConA and A23187 differed in their ability to induce dbl neg thymocytes to produce IL-2. At present we do not know why ConA failed to elicit IL-2 from dbl neg thymocytes. One possibility is that these cells do not yet express the high affinity ConA receptors (probably the antigen-binding receptors themselves) that can transduce ConA binding into a mitogenic signal. This issue is being investigated.

In a separate series of experiments, we tested day 15 (D15) fetal thymocytes for their ability to produce IL-2 in response to A23187 + TPA. These studies were prompted by recent findings that D15 fetal and young adult dbl neg thymocytes are phenotypically similar (Ceredig et al., 1985; Takacs et al., 1984). However, in our culture system D15 fetal thymocytes failed to produce IL-2. This failure could not be ascribed to the absence of Ly-1+ cells, presumably destined to become medullary cells (Scollay and Shortman, 1985), in the D15 fetal population, because young adult dbl neg thymocytes from which the Ly-1+ cells had been removed continued to produce IL-2 in response to A23187 + TPA. These results demonstrate that D15 and young adult dbl neg thymocytes are developmentally distinct.

References:


1 Undergraduate, California Institute of Technology.

203. In vitro Culture of Precursor T Cells

James P. Lugo, Ellen Rothenberg

During our stimulation experiments using A23187 and TPA, we observed that dbl neg thymocytes underwent blast transformation in vitro, turning their culture medium bright yellow. This observation prompted us to reculture these cells in media containing 20% culture supernatants from ConA-stimulated spleen cells. To our surprise (and delight), the surviving cells began to grow at a steady rate, doubling five times over the next four days. After two days of maintenance culture (three days in vitro overall), the cells were still >95% dbl neg and >90% Thy-1+. Following another round of stimulation and two more days of growth, the population became more complex. Lyt-2+ cells now accounted for about 15% of the population, and cells bearing TL (thymus-leukemia antigen, a marker of the cortical lineage) appeared (~10%). The cultures also included very large cells with low Thy-1 expression. These results indicate that pre-T cells with the capacity to differentiate can survive in appreciable numbers through at least five doublings in vitro. More recently, we found that D15 fetal and Thy-1+ thymocytes will also grow in culture using this approach.

Over the coming year, different culture conditions and stimulation regimens will be assayed with the aim of optimizing cell growth, particularly those cells retaining the dbl neg phenotype. Initially, we will also try to maintain some fraction of continuously differentiating cells, i.e., Lyt-2+, L3T4- or Lyt-2-, L3T4+ cells, with or without concomitant appearance of Lyt-2+, L3T4+ cortical-type cells. This will provide some assurance that the proliferating cells retain the potential to differentiate. Ultimately, however, we will reintroduce these cells back into the thymus in order to measure their full ontogenetic potential. Two approaches will be used: injection into sublethally (Fowlkes and Mathiesen, 1984) or lethally (Hirokawa et al., 1985) irradiated animals, or injection into thymus lobes maintained in culture and depleted of endogenous thymocytes by 2-deoxyguanosine treatment (Jordan et al., 1985; Ready et al., 1984). Attempts can also be made to assess the effects of various thymic hormones on these cells, or any cloned cell lines that we derive from them. Parallel studies using 5-azacytidine and
retinoic acid are also planned.

References:

204. A Suicide Response in Major Cortical Thymocytes

*James P. Lugo, Ellen Rothenberg*

PNA+ thymic lymphoblasts and cortical cells undergo rapid cell death in response to treatment with A23187 + TPA. This response is fundamentally different from all the other thymocyte populations we have tested thus far, including extremely primitive Thy-1- cells. Stimulation for various times and with various concentrations of A23187 failed to induce these cells to make IL-2. Furthermore, those concentrations and incubation periods that yielded optimal amounts of IL-2 from mature T cells, dbl neg and PNA- thymocytes consistently caused the PNA+ blasts to die rapidly. ConA + TPA, by contrast, did not have as startling an effect on this population. Cohen and Duke (1984) have shown that thymocytes contain a calcium-dependent endonuclease whose specificity is similar to that of micrococcal nuclease. They also found that this DNase activity is induced by corticosteroids and A23187, and is inhibited by 5 mM Zn++ and cycloheximide treatment. Since they employed unfractionated thymocytes in their studies, their observations are most relevant to PNA+ cells, which comprise the bulk (85-90%) of thymic lymphocytes. We plan to stimulate PNA+ cells with A23187 + TPA in the presence of cycloheximide in order to assess the ability of these cells to transcribe the IL-2 and IL-2R genes. RNA will be detected in individual cells by *in situ* hybridization and in the population overall by probe protection. Furthermore, we will examine the ability of these cells to respond to ConA by determining whether lectin binding induces a calcium flux to occur within these cells. Bulk population measurements will be carried out in a spectrofluorimeter using the fluorescent calcium indicator fura-2 (Grynkiewicz *et al.*, 1985); single cell measurements, aimed at assessing the population's response heterogeneity, will be carried out by flow cytometry using indo-1 (Grynkiewicz *et al.*, 1985). These studies will help to characterize more fully the developmental status of PNA+ thymocytes.

References:

205. Transcription of T-Cell Receptor α- and β-Chain Genes in Subpopulations of Adult Mouse Thymocytes

*Christine Kinnon, Rochelle A. Diamond, Ellen Rothenberg*

T-cell maturation and development are thought to involve the selective amplification and destruction of pre-T-cell clones. The mechanisms that govern these processes are currently unknown; however, results from our laboratory have suggested that thymocytes commit themselves to alternative developmental fates while undergoing proliferation in the thymus. The selective expression of T-cell receptors by some cells in the thymus during development may play a role in determining their ultimate fates. In this study, we have analyzed the expression of T-cell receptor genes at the RNA level in subpopulations of adult thymocytes. In particular, we have separated two populations of large proliferating blast cells from the vast majority of small nondividing cortical thymocytes, using a combination of size fractionation and selection on the basis of binding to the lectin peanut agglutinin (PNA). These cells were examined for the differential expression of two of the three currently known T-cell receptor genes by RNA blot analysis using cDNA probes encoding the α and β chains of the T-cell receptor, which were kindly provided by Prof. Lee Hood's lab (Caccia *et al.*, 1984; Winoto *et al.*, 1985). Our results have shown that the nondividing postmitotic cells express high levels of mRNA for both α and β chains. Small cortical thymocytes, in spite of their functional incompetence, express the highest concentrations of full-length α-chain mRNA of any cells in the thymus. The blast cell populations overall express about 10-fold less α-chain transcripts relative to their levels of β-chain mRNA. Even so, the blast cells apparently committed to the cortical lineage express α mRNA at essentially the same concentration.
as mature medullary cells. These results imply that the cortical cells are not grossly deficient in the rearrangement or transcription of α-chain genes. The reasons for their death must lie elsewhere.

References:

206. Expression of T-Cell Receptor Genes by Subpopulations of Immature Precursor Cells in the Adult Thymus

Christina Kirnon, James P. Lugo, Santosh N. Krishnan, Ellen Rothenberg

We have isolated highly immature "double-negative" (dbl neg) cells (Ly-2-, L3T4-, Thy-1-) from adult thymus using the technique of cytolytic elimination (see Abstract Nos. 200, 202 and 203). This population of cells represents a very small proportion of adult thymocytes (~3%) and should include precursor cells for all other classes of thymocytes. Since it is unclear when T-cell receptors are first expressed by developing thymocytes, it is important to fully characterize this precursor cell population. We have investigated T-cell receptor expression at the level of steady-state RNA concentrations by RNA blot analysis using α-, β- and γ-chain probes obtained from Prof. Lee Hood's lab (see previous abstracts also A. Winoto, unpublished observations). We have found that while β-chain mRNA is expressed at levels similar to those observed in small cortical thymocytes (~80% of total thymocytes), α-chain mRNA is expressed at much lower levels (~10 fold).

A third gene, γ, which has remarkably similar properties to the α- and β-chain T-cell receptor genes, may encode a third chain of the T-cell receptor. This gene appears to be expressed by mature cytotoxic T cells (Saito et al., 1984; Kranz et al., 1985). γ-Chain mRNA also is expressed at much higher levels in this population than in other thymocytes, as it is undetectable in other thymocyte populations. These results agree with those of others (Raulet et al., 1985).

DbI neg cells also include the only detectable cells in the thymus that express IL-2R (see Abstract No. 200). We are currently attempting to compare T-cell receptor expression in these cells with expression in other proliferating thymocytes and in dbl neg cells that do not express IL-2R. This is particularly interesting because mature T cells that proliferate in response to antigen invariably express IL-2R. Thymocytes that lack IL-2R may proliferate by a mechanism that is independent of antigen stimulation, and may not therefore depend on a functional T-cell receptor (Lugo et al., 1985).

References:

1 Undergraduate, California Institute of Technology.

207. Analysis of Thymocyte Gene Expression by in situ Hybridization

Kathleen L. McGuire, Ellen Rothenberg

We are interested in developing the technique of in situ hybridization to further investigate the developmental processes occurring in the mammalian thymus. This technique, which will utilize labeled single-stranded cRNA probes to detect cytoplasmic RNA, enables the investigators to analyze the mRNA of individual cells in a suspension or tissue section. This distinguishes between all the cells in a population producing a particular message, and a fraction of that population producing all of the message. The in situ hybridization technique offers the added capability of determining the anatomical distribution of cells producing a particular mRNA in a tissue section. Neither of these determinations are possible with conventional RNA blot hybridization.

Previous results from this laboratory (Caplan and Rothenberg, 1984; also see Abstract No. 202) demonstrated that certain populations of adult thymocytes are capable of producing IL-2 when stimulated with ConA or a calcium ionophore and the phorbol ester TPA. These populations include some cells with immature phenotypes. The in situ hybridization technique will allow us to determine if the immature (dbl neg) population which produces IL-2 is homogeneous or whether only a subpopulation of these cells is respon-
sible for the production seen. Similarly, we can use this technique to probe the physiological significance of this IL-2 production in the development of the thymus by determining if the IL-2 gene is transcribed by unstimulated thymic lymphoblasts. Subsequently, we will analyze tissue sections of thymuses isolated from normal and antigen-primed mice in an effort to determine the anatomical distribution of the IL-2-producing thymic lymphoblasts.

Additional studies using this technique are planned to further study the development of the murine thymus. We are currently subcloning α, β and γ T-cell receptor cDNAs (provided by the laboratory of Prof. Lee Hood) into appropriate vectors (C. Kinnon and K. McGuire, work in progress) to enable us to synthesize cRNA probes. With these, we will attempt to elucidate the phenotype and location of thymocyte populations capable of transcribing these three genes. Furthermore, this technique can be utilized to study the expression of other genes, such as oncogenes and hormone receptor genes, to investigate their role in the proliferation and differentiation processes of the mammalian thymus.

208. A T-Cell Genomic Library with a Selectable Transformation Marker

Christine Kinnon, Ellen Rothenberg

A genomic library was prepared in the cosmid expression vector pCV108. This vector contains the selectable marker SV2-neo, which will allow us to transform mammalian cells directly with recombinant cosmids of interest isolated from this library, and identify transformants by resistance to G418 without further manipulation (Lau and Kan, 1983). High molecular weight DNA prepared from the murine helper T-cell thymoma line EL4.E1 was partially digested with the restriction endonuclease Sau3A and size-fractionated on a sucrose gradient. Following ligation of the insert DNA to linearized, dephosphorylated cosmid vector DNA, the recombinant cosmids were packaged and used to transduce E. coli 490A. The library (about 3 x 10^5 recombinant clones) was plated onto Biodyne membrane filters and screened with an IL-2 cDNA probe.

Reference:

209. Stable Transformation of T Cells by DNA-mediated Transfection

Thomas J. Novak, Ellen Rothenberg

Our goal of studying T cell-specific gene expression required that we develop a procedure for introducing exogenous DNA efficiently and stably into a recipient T cell's genome and that this DNA be expressed. To this end we have successfully used the bacterial aminoglycoside phosphotransferase (neo) gene to transform mature murine and human T-cell lines to G418 resistance. The selection system used was described in last year's Annual Report, and has been used to test the E. coli neo gene from Tn5 under the control of three eukaryotic promoters: 1) the RSV LTR; 2) the SV40 early promoter, and 3) the HSV TK gene promoter.

As described in last year's Annual Report, the only DNA delivery system that works consistently well in our hands is protoplast fusion. Neither Ca_3(PO_4)_2 precipitation nor polycation facilitation yielded a positive result. When introduced into an adherent murine cytotoxic T-cell line, MTL.2.8.2, all three constructs yielded a similar frequency of transformation of ~1 x 10^-4. Transformation was scored as the ability of cells to grow for ≥4 weeks in 1 mg/ml G418. Mock transfected cells generally die in 2-3 weeks.

A murine T-cell thymoma line, EL4.E1, is transformed at a lower frequency of ~3.3 x 10^-6 with the RSV- or SV2-based constructs; no TK-neo derived transformants have been isolated from this cell line. The lower transformation frequency seen for this line is probably due to several factors including: 1) less efficient expression of exogenous DNAs in these cells (see next abstract); 2) nonadherent growth, which inevitably results in cell loss during feeding; and 3) the extreme variability in G418 sensitivity in these cells.

We have also examined a human IL-2-dependent T-cell line called MJ. Initial experiments demonstrated a frequency of transformation of well over 10^-4 for all three constructs. Dot blots of cell DNA from randomly pooled microtiter wells were all positive for neo sequences. However, the cells transformed with RSV- or SV2-based constructs contained 10X more neo DNA than those transformed with the TK construct.

Southern blot analysis of genomic DNA from transformed murine T-cell lines showed that in all cases
except one, the number of copies of neo sequences per cell was less than one. The lone exception contained 1-2 copies per cell. We interpret this as evidence for the instability of the integration event. Further evidence of this instability is provided by several MTL transformants that have been growing in bulk culture in 1 mg/ml G418 for more than 6 months. One of these six lines contained detectable neo sequences (>0.2 copies/cell) when analyzed. These lines grow slowly in tight, discrete foci, which distinguish them from normal cells. We suspect that the low copy number needed to confer G418 resistance coupled with the high tolerance of MTL cells for G418 (5-10 times less sensitive than our other lines) permit cross-feeding between cells and the retention of non-transformants and/or revertants in our cultures. We are presently selecting for primary MTL transformants with the aim of immediately subcloning cells that exhibit G418 resistance. It is hoped that a more vigorous selection early in the transformation cycle will result in a greater frequency of cells that retain the selected gene.

210. T Cell-specific Responses to Transcriptional Regulatory Sequences

Thomas J. Novak, Ellen Rothenberg

One of the ultimate goals of this lab is the introduction into T cells of cloned genes linked either to their own promoters or to those of other genes. Therefore, simultaneous with our efforts to establish a stable transformation system for T cells, we carried out a series of experiments to evaluate the strengths of a variety of eukaryotic promoter constructs. Briefly, MTL, EL4 or L cells were transfected with test plasmids by protoplast fusion. Two days post-transfection, cells were harvested and lysed and these cellular extracts were assayed for the presence of the recorder gene product. Additionally, nuclei were isolated from the lysed cell pellets and DNA was extracted for an estimation of recorder gene copy number by quantitative DNA dot blots.

The chloramphenicol acetyltransferase (CAT) assay results suggested striking differences in the strengths of these promoters in T cells, counter to our observation of equal transformation efficiencies with each of the three neo constructs. The disparity could be due to the different readout systems used. A weak prepared from cells two days posttransfection are run on a nondenaturing 10% acrylamide gel. Following electrophoresis, the gel is submerged in buffer and incubated with γ-32P-ATP and kanamycin or neomycin, the cofactor and substrate, respectively, for the phosphotransferase reaction. The enzymatic reaction occurs within the gel matrix and the phosphorylated kanamycin is then blotted out of the gel and onto P81 cation exchange paper where it can be detected by autoradiography. The authenticity of the gene product can be verified by running, in parallel, extracts from mock transfected cells and extracts from bacteria harboring plasmids with the Tn5 transposon. Though less sensitive and less convenient than the CAT assay, this assay has, nonetheless, provided useful information regarding the hierarchy of promoter strengths in T cells.

Our CAT assay results indicated promoter hierarchy in both T cell lines of RSV>SrM2-SV2>TK. In L cells the hierarchy was SrM2-RSV>SV2>TK, with a relatively greater signal from the SrM2 construct. The SrM2 promoter is identical to the SV40 early promoter except that SV40 enhancer has been replaced by the 72 base pair repeat enhancer element from Moloney murine sarcoma virus.

The T cells did not show a preferential response to enhancer sequences derived from the immunoglobulin heavy chain gene, which strongly increase gene activity in B cells (Banerji et al., 1983; Gillies et al., 1983). Thus their responses appear to be distinct both from those of fibroblasts (L cells) and from those of other types of lymphocytes.

References:

211. Effect of Coding Sequences on Relative Promoter Strength in T Cells

Thomas J. Novak, Ellen Rothenberg

The chloramphenicol acetyltransferase (CAT) assay results suggested striking differences in the strengths of these promoters in T cells, counter to our observation of equal transformation efficiencies with each of the three neo constructs. The disparity could be due to the different readout systems used. A weak
promoter might make enough aminoglycoside phospho-transferase to allow selection in G418 but might not produce enough CAT to score positively. To test this possibility we measured transient expression of neo. The relative promoter strengths in the neo assay, normalized for DNA copy number, were as follows:

<table>
<thead>
<tr>
<th></th>
<th>MTL</th>
<th>EL4</th>
<th>L Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>0.43</td>
<td>0.16</td>
<td>0.51</td>
</tr>
<tr>
<td>SV2</td>
<td>1.0</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>TK</td>
<td>0.01</td>
<td>0.01</td>
<td>0.05</td>
</tr>
</tbody>
</table>

L cell and MTL values represent means of three experiments. EL4 values represent means of two experiments.

Taking together the CAT and neo assay data, it is clear that the comparative strengths of the SV40 promoter and the RSV LTR differ drastically depending on the gene that they are driving. Whereas the RSV LTR drives the expression of CAT at least 50 times more efficiently than SV40, the SV40 promoter is comparable or stronger when the promoters are linked to the neo gene. Surprisingly, the SV2-neo construct that yields the most L cell stable transformants (data not shown) is expressed one order of magnitude less well than the RSV construct in the same cells. However, as this level is still higher than the TK construct in MTL cells, it may be more than sufficient to confer G418 resistance.

### PUBLICATIONS


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Research Staff: Venice L. Radice, Joan L. Roach
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Summary: We are interested in the molecular dynamics underlying neuronal function. Central nervous system neurons respond to a variety of chemical and electrical stimuli that reflect the state of the neural circuits around them, as well as the state of the animal as a whole. One of the ways that external stimuli affect the neuron is by inducing changes in the intracellular concentration of calcium ion. These changes alter many cellular processes and properties, including neurotransmitter synthesis and release, axonal transport, membrane excitability, cell shape, cell motility and, in some cases, formation and maintenance of functional synaptic connections. Several of these subtle forms of regulation are believed to be important for learning and formation of memory. The biochemical effects of calcium ion that underlie these functional changes are correspondingly complex and are not yet completely understood. An intracellular calcium-binding protein called calmodulin regulates the functions of many neuronal proteins.

We have purified and characterized rat brain Type II Ca$^{2+}$/calmodulin-dependent protein kinase. This newly described class of protein kinases is far more highly expressed in brain than in other tissues, constituting ~0.7% of total brain protein. It is also more highly expressed in telencephalic neurons, such as those in the hippocampus and cortex, than in neurons in lower brain regions. The rat brain enzyme is a large oligomer composed of approximately 12 structurally-related 50,000 and 60,000 dalton subunits, which are expressed in different ratios in different brain regions. For example, the forebrain isozyme contains about nine α-subunits and about three β-subunits, while the cerebellar isozyme contains about two α-subunits and about eight β-subunits.

We are turning our attention to learning what functions are regulated by the kinase and its subunits. We are investigating the locations of the kinase subunits within neurons and their interactions with the cytoskeleton and subcellular organelles. For example, we have found that in the forebrain, where the holoenzyme contains mostly α-subunits, the kinase is a prominent component of "postsynaptic densities," fibrous organelles that lie beneath the postsynaptic membrane. In the cerebellum, where the holoenzyme contains mostly β-subunits, it is not so enriched in postsynaptic densities but is associated with other particulate structures. We have unraveled the complex regulation of kinase enzymatic activity by its own autophosphorylation. This mechanism produces an amplification of the calcium signal, and provides for a low level of kinase activity that can outlast the calcium transient, and must be turned off by cellular phosphatases. We also are beginning to select cDNA probes coding for the kinase subunits. These can be used for structural studies, for identifying homologous proteins in lower animals in which genetic and physiological studies are possible, and perhaps eventually for examining the functions of the kinase in mammalian cells by manipulating expression of the kinase subunits in cells in culture.

212. Modulation of the Catalytic Activity of Type II CaM Kinase by Autophosphorylation

Stephen G. Miller

The α-subunit (50,000 daltons) and β-subunit (60,000 daltons) of Type II CaM kinase are rapidly autophosphorylated at multiple sites in the presence of Ca$^{2+}$ and calmodulin. The kinase can incorporate ~3 moles phosphate per mole α-subunit and ~2 moles per mole α-subunit. We have investigated the possible effects of autophosphorylation on the catalytic activity of the kinase.

Purified forebrain kinase was autophosphorylated with cold ATP in the presence of Ca$^{2+}$ and calmodulin, or in the presence of EGTA to chelate Ca$^{2+}$. The kinase was then diluted 500 fold and assayed for enzymatic activity in the presence or absence of Ca$^{2+}$ with synapsin I as substrate. We observed two effects of autophosphorylation on enzymatic activity. 1) The initial rate of synapsin I kinase activity was reduced to ~20-30% of control levels. 2) A large fraction of this
activity had become independent of Ca\(^{2+}\) and calmodulin. The control kinase showed no Ca\(^{2+}\)/calmodulin-independent activity. Thus, autophosphorylation produced a significant level of Ca\(^{2+}\)-independent activity while at the same time making the kinase refractory to additional Ca\(^{2+}\) stimuli.

The change in initial rate of synapsin I phosphorylation in the presence of calcium was due to a decrease in \(v_{\text{max}}\) while the apparent \(K_{m}\) remained the same. An identical change in kinase activity occurred when microtubule-associated protein 2 (MAP2) was used as a substrate. The incorporation of as few as three to five phosphates per holoenzyme (much less than one per subunit) was sufficient to cause maximal shift in kinase activity.

The physiological implications of this autophosphorylation may be important in the regulation of synaptic function. In vivo, this mechanism could produce Ca\(^{2+}\)-dependent bursts of high kinase activity followed by a change in state in which the kinase is refractory to further calcium stimuli and maintains a low level of calcium-independent activity. Return to the initial calcium-sensitive state would require dephosphorylation by cellular phosphatases.

213. Autophosphorylation of Type II CaM Kinase in the Absence of Ca\(^{2+}\)

Stephen G. Miller

The appearance of a Ca\(^{2+}\)-independent activity after low levels of autophosphorylation led us to investigate the effects of autophosphorylation on further incorporation of phosphate into the kinase itself. We found that rapid Ca\(^{2+}\)-independent autophosphorylation was initiated by the Ca\(^{2+}\)-dependent autophosphorylation of only a few subunits per holoenzyme.

Only extremely low rates of autophosphorylation of native (dephospho) kinase occur in the absence of Ca\(^{2+}\). However, when the kinase was incubated briefly in the presence of Ca\(^{2+}\) and calmodulin and then EGTA was added to chelate Ca\(^{2+}\), autophosphorylation continued at nearly the same rate in the absence of Ca\(^{2+}\) as in its presence. The final number of phosphates incorporated per mole of kinase was about 30% higher in the presence of Ca\(^{2+}\), suggesting that some of the autophosphorylation sites may be strictly Ca\(^{2+}\)-dependent. Multiple site phosphorylation may extend the lifetime of the Ca\(^{2+}\)-independent state described in Abstract No. 212 by increasing the time required for removal of phosphate by phosphatases.

The rate of Ca\(^{2+}\)-dependent autophosphorylation of native kinase was linear over an 80-fold range of kinase concentration, indicating that this reaction is primarily intramolecular. We wanted to know whether the Ca\(^{2+}\)-independent phosphokinase was capable of activating native kinase by an intermolecular autophosphorylation. Kinase that had been maximally phosphorylated with cold ATP was incubated with native kinase at various ratios in the presence of labeled ATP and in the absence of Ca\(^{2+}\)/calmodulin. Under these conditions, apparent intermolecular autophosphorylation occurred at \(<5\%\) of the intramolecular rate. No shift in the activity of the native kinase was observed. We consider it unlikely, therefore, that phosphokinase is able to propagate its activated state through the cell by triggering Ca\(^{2+}\)-independent activity of native kinase in vivo.

214. Association of Type II CaM Kinase with the Postsynaptic Density Fraction in Rat Forebrain and Cerebellum

Stephen G. Miller

The postsynaptic density (PSD) was originally described as a fibrous, electron-dense structure underlying the postsynaptic membrane at most CNS synapses. Our group and others have shown that the major polypeptide component of purified PSD fractions (mPSDp) is the \(\alpha\)-subunit of the Type II kinase. Several groups have investigated the protein composition of PSD fractions obtained from different brain regions and have shown that the PSDs isolated from cerebellum contain only small amounts of the mPSDp, now known to be the \(\alpha\)-subunit. We have determined which form (forebrain or cerebellar) of the Type II kinase is associated with the PSD fractions isolated from these two brain regions and have quantitated the amount of kinase in each fraction.

PSD fractions were isolated from rat forebrain or cerebellum by the method of Carlin et al. (1980). Comparison of endogenous phosphorylation patterns of freshly prepared PSDs to the autophosphorylation patterns of pure Type II CaM kinase from each brain region suggested that the PSD fraction from each region contained the corresponding isozyme of the
Type II kinase. The molecular weights and relative proportions of the major endogenous phosphotides were identical to the autophosphorylated subunits of the pure enzymes from the corresponding brain regions. However, the cerebellar PSD fraction appeared to contain reduced quantities of both subunits when compared to the forebrain PSD fraction.

We used a quantitative immunoblot procedure to measure the content of each kinase subunit in PSD fractions from the two regions. We used a polyclonal antisera specific to the α-subunit and a monoclonal antibody specific to the β-subunit. We found that the forebrain PSDs contain a ratio of α- to β-subunits of about 3.5:1, which together comprise about 16-18% of total PSD protein. In contrast, the cerebellar PSDs contain α- and β-subunits in an approximate ratio of 1:1, which together make up only about 1-2% of total cerebellar PSD protein. Thus, the PSD fraction from each brain region contains the isozyme of the Type II kinase specific for that region; but, the cerebellar PSD fraction contains much less total kinase than the forebrain PSD fraction. This implies that cerebellar PSDs may produce quantitatively different responses to synaptic activation than those in the forebrain.

Reference:

215. Association of Type II CaM Kinase with Brain Microtubules

Ngozi E. Erondu

It has been shown in a number of laboratories including ours that microtubule-associated proteins (MAPs) and tubulin can be phosphorylated by a calcium/calmodulin-dependent protein kinase. This phosphorylation also has been shown to cause an inhibition of microtubule assembly in vitro (Yamamoto et al., 1985). While a tubulin-associated, calmodulin-dependent kinase has been purified from rat brain (Goldenring et al., 1984), no detailed structural or functional characterization of the association between the kinase and microtubules (MTs) has been carried out. We are therefore interested in defining in a quantitative manner the nature of this interaction. We would also like to determine what factors regulate it and the functional consequences of such an association.

In our initial experiments, we isolated MTs from rat brain cytosol using the assembly-promoting drug taxol. We recovered about 2.5% of total enzyme activity in the microtubule pellet, but there was no enrichment of the specific activity of the kinase. However, when the isolation was carried out in the presence of calcium and calmodulin, there was a 5- to 10-fold enrichment of kinase activity in the MT pellet. Subsequently, we have examined the association using pure MTs and pure kinase. MTs isolated by two cycles of assembly/disassembly (2 x MTs) were polymerized with taxol in the presence of pure kinase. The proportion of activity recovered in the pellet increased approximately 10-fold in the presence of pure kinase. The proportion of activity recovered in the pellet increased approximately 10-fold in the presence of calcium and calmodulin. This increased association required both calcium and calmodulin. A Scatchard analysis indicated a single binding site with an apparent $K_D$ of 44 nM. Assuming that the MTs contain tubulin dimer and MAP$_2$ in a molar ratio of 9:1, there is one mole of kinase binding site per 150 moles of tubulin dimer and 17 moles of MAP$_2$. This compares to one mole of binding site for cAMP-dependent protein kinase per 40 moles of MAP$_2$ (Theurkauf and Vallee, 1982). We are presently examining the possibility that this increased association in the presence of Ca$^{2+}$ and calmodulin is a consequence of autophosphorylation of the enzyme (cf. Abstract No. 212). We would also like to determine which components of the MTs and which subunit of the kinase are involved in the interaction.

References:

216. Development of a Radioimmunoassay for Brain Type II CaM Kinase

Ngozi E. Erondu

We have previously described the use of a solid-phase immunoassay to study the distribution of the α-subunit of Type II CaM kinase in rat brain (Erondu and Kennedy, 1985). We have now used two precipitating monoclonal antibodies (4A11 and 6E9) to develop a liquid phase radioimmunoassay. This assay complements the solid phase assay in a number of ways: a) it measures the holoenzyme in its native
form; b) it permits the assay of more samples in a shorter period of time; and c) it requires lower quantities of most of the reagents.

Pure kinase is radioiodinated by the Bolton-Hunter procedure. This results in the incorporation of one mole of \(^{125}\text{I}\) per three moles of kinase—a specific radioactivity of 1000 Ci/mmol. Assay tubes contain increasing amounts (0 to 200 ng) of pure kinase, antibody and 10 ng (10,000 cpm) \(^{125}\text{I}\)-kinase. The amount of antibody is sufficient to precipitate 40-50% of labeled enzyme. After a two-hour incubation on ice, rabbit anti-mouse IgG serum (purified on a mouse IgG column) is added, and the incubation is continued for another hour. Protein A bearing Staphylococcus aureus cells are then added, and the incubation is continued for 30 minutes. Immune complexes are pelleted in a microfuge, washed, and counted in a gamma counter. The data are plotted on a log-logit scale, and the standard curves are linear between 2.0 and 200 ng kinase. The limit of detection is 2.0 ng (p <0.05).

With these assays, it became evident that monoclonal antibody 6E9 does not recognize phosphokinase while 4A11 recognizes phosphokinase poorly. We are presently trying to develop a similar assay using an antibody that recognizes both phosphorylated and unphosphorylated forms of the enzyme. With a combination of these assays, we will be able to determine the proportion of the enzyme that is present in either form and determine whether this proportion changes under a number of physiological conditions.

Reference:

217. Characterization of cDNA Clones for the Subunits of Brain Type II CaM Kinase

Mark K. Bennett

A number of important questions about the structure of the kinase holoenzyme and the relatedness of the subunits can best be approached at the nucleic acid level. We have isolated two cDNA clones from a \(\lambda gt11\) expression library that code for the kinase (Biology 1984, Abstract No. 178). The clones have inserts of 1.0 and 0.6 kilobases (kb). Analysis of the hybrid proteins indicates that the sequence of both cDNAs is nearly entirely coding. Both cDNAs have been subcloned into the single-stranded bacteriophage vectors M13mp18 and M13mp19. Restriction endonuclease mapping indicates the 0.6 kb insert is contained within the 1.0 kb insert. For this reason, only the 1.0 kb clone has been characterized further.

In order to identify which subunit the cDNA codes for, the single-stranded M13 subclone was used to hybrid select rat brain messenger RNA. The selected RNA was translated in vitro and the products of in vitro translation were immunoprecipitated with a monoclonal antibody and analyzed by SDS-polyacrylamide gel electrophoresis. The 1.0 kb insert was able to hybrid-select mRNA for both subunits of the kinase when inserted in such a way that the single-stranded form of the clone was anticoding. When inserted into M13 in the opposite orientation (single-stranded form coding), the clone did not select message for the kinase subunits. Peptide mapping studies of the kinase subunits indicate many structural similarities. This hybrid selection result may reflect the similarity of the two subunits at the mRNA level.

Northern blot analysis indicates that the 1.0 kb insert hybridizes with a 5.0 kb message in brain poly(A)+ RNA that is more abundant in cerebellum than in forebrain. Thus, the clone may code for the \(\beta\)-subunit, which is more highly expressed in cerebellum. No reaction was found on Northern blots using brain poly(A)+ or liver poly(A)+ RNA.

218. Protein Sequence Analysis and Generation of Oligonucleotide Probes for Brain Type II CaM Kinase

Mark K. Bennett, James B. Hurley

A second approach we have used to obtain cDNA clones coding for the kinase subunits is to determine the amino acid sequence for a particular subunit and use that sequence data to synthesize oligonucleotides. These oligonucleotides can then be used to screen cDNA libraries. The amino terminals of both subunits were found to be blocked, both in a native state and as isolated from SDS gels. Therefore, we decided to look for proteolytic fragments of the kinase subunits that could be sequenced. We have isolated, by HPLC gel filtration, a 20,000 dalton peptide from a chymotryptic digest of native kinase. The peptide, based on its size and abundance, appears to have been derived from the \(\alpha\)-subunit of the kinase. The sequence of the first 20 amino acids from the amino terminal of this peptide
has been determined. Two mixtures of oligonucleotides corresponding to partially overlapping regions of the sequence were prepared by Ms. Marilyn G. Tomich and Dr. Suzanna Horvath. One is a mixture of thirty-two 17 mers and the other is a mixture of forty-eight 17 mers.

Both of the oligonucleotide probes react on Northern blots with a 5.3 kb message from brain poly(A)+ RNA but not brain poly(A)- or liver poly(A)+ RNA. The identification of these probes as α-subunit-specific takes advantage of the fact that the α-subunit is much more abundant in the forebrain than in the cerebellum. In vitro translation of forebrain and cerebellar RNA followed by immunoprecipitation with a monoclonal antibody has shown that the mRNAs for the two subunits also have an uneven distribution between forebrain and cerebellum. The α-subunit mRNA is much more abundant in the forebrain. Both oligonucleotide probes react on Northern blots with a message that is more abundant in forebrain than in cerebellum, thus verifying that the probes are α-subunit specific.

The oligonucleotides were used to screen two λgt10 rat brain cDNA libraries generously provided by David Anderson of Columbia University and Arthur Roach of Lee Hood’s laboratory. A total of 500,000 phage were screened and no clones were found to give consistently positive signals with both probes. The inability to detect positives may be the result of a large 3' untranslated region on the message, which makes it difficult to obtain full length cDNAs. The oligonucleotides will be used to screen libraries with larger inserts. They will also be useful in the construction of a new cDNA library.

219. Type II CaM Kinase System in Drosophila

John Wall1, Venise L. Radice

Homogenates of Drosophila heads contain a calcium- and calmodulin-dependent protein kinase with many characteristics of the mammalian Type II CaM kinase. It is more concentrated in head homogenates (2.1 nmols/min/mg) than in body homogenates (0.3 nmols/min/mg), suggesting that it is concentrated in nervous tissue. It phosphorylates synapsin I and is inhibited by a monoclonal antibody that was raised against and inhibits the rat brain Type II CaM kinase. This antibody can be used to specifically immunoprecipitate two protein bands, which appear to be the Drosophila kinase subunits. They comigrate with the β- and β'-subunits of the rat brain enzyme. Like the rat brain subunits, they can be endogenously phosphorylated in head homogenates. The presence of this apparently homologous protein kinase system in Drosophila opens the way for genetic studies on its functions. In collaboration with William Quinn at MIT, we are examining a series of learning mutants for defects in the kinase system.

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220. Denervation of Monkey Cortical Neurons Alters Their Immunocytochemical Staining for Type II CaM Kinase

Stewart H. Hendry1, Mary B. Kennedy

We have used a horseradish peroxidase linked immunocytochemical technique, based on binding of monoclonal antibody 6G9, to label neurons in the monkey visual cortex that contain the α-subunit of Type II CaM kinase. Stained neurons are densely packed in layers II and IVB and are also contained in layers III, IVCa and VI. In normal animals, the distribution of kinase-positive cells within a layer is relatively uniform. However, in animals in which one set of ocular dominance columns has been denervated by removal of an eye 7-14 days before sacrifice, layer IVCa is divided into alternating lightly and darkly staining bands. Comparison of the sections with adjacent sections stained for the mitochondrial enzyme, cytochrome oxidase, reveals that the kinase staining increases in cell bodies in the denervated ocular dominance columns. Immunocytochemical staining for synapsin I, a major presynaptic substrate for the kinase, remains uniform under these conditions. We interpret the findings to suggest that the concentration of Type II CaM kinase increases in neurons in the visual cortex when they are deprived of their normal visual input. This suggests that neuronal activity may be able to regulate the level of expression of the Type II CaM kinase in postsynaptic neurons. Thus, activity may alter the regulatory machinery of neurons over time.

1Department of Neurobiology and Anatomy, California Medical School, University of California, Irvine.
concern the gating of ion channels and their regulation. Progress has now been made on serotonin receptors and on Ca channels. (3) We want to understand the structure and function of channels by observing the properties of channels that have been mutagenized at known amino-acid residues. For the present, this final goal is being conducted at nicotinic acetylcholine receptors.

These studies have required the development of modern patch-clamp methods for *Xenopus* oocytes. They also require a fuller understanding of the role of intracellular messenger systems in these cells.

This year saw the end of our decade-long studies on nicotinic acetylcholine receptors using flash-activated molecules. The light-flash approach has also been used to study kinetic aspects of intracellular transmitters and channel blockers, and we have now conducted experiments with a molecule that releases Ca\(^{2+}\) in response to irradiation.

The Pine group, informally associated with this laboratory, continues to be interested in the application of sophisticated techniques to the study of neuronal development. Microcircuit culture dishes are being improved in reliability; voltage-sensitive dyes and channel blockers, and we have now conducted experiments with a molecule that releases Ca\(^{2+}\) in response to irradiation.

The experiments fall into three classes. (1) We exploit the fact that these channels are expressed in a new context, where it is often possible to study them, and their modulation by neurotransmitters and intracellular messengers, more directly than in the cells of origin. Useful information is being gained about GTP- binding proteins in intracellular signaling. (2) We want to use expression in *Xenopus* oocytes as an assay for RNA fractions, or for cDNA clones, encoding the channels. Progress has now been made on serotonin receptors and on Ca channels. (3) We want to understand the structure and function of channels by observing the properties of channels that have been mutagenized at known amino-acid residues. For the present, this final goal is being conducted at nicotinic acetylcholine receptors.

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The Pine group, informally associated with this laboratory, continues to be interested in the application of sophisticated techniques to the study of neuronal development. Microcircuit culture dishes are being improved in reliability; voltage-sensitive dyes are also being used in parallel with these dishes. Soft X-ray microscopy is becoming a reality, with the help of better apparatus and signal processing.

**PUBLICATIONS**


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**Professors:** Henry A. Lester, Jerome Pine

**Visiting Professor:** Douglas M. Fambrough

**Senior Research Fellow:** Jeanne M. Nerbonne

**Myron A. Bantrell Research Fellow:** Nathan Dascal

**Research Fellows:** Lee D. Chabala, Alison M. Gurney, Helen Rayburn\(^1\), Michael M. White

**Graduate Students:** Chi-Bin Chien\(^1\), John R. Gilbert\(^1\), Arie M. Michelsohn, Bruce L. Patton, Brian Rasnow\(^1\), Wade Regehr\(^2\)

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- System Development Foundation

**Summary:** This past year has seen major changes in the research program of our group. Our interests still concern the gating of ion channels and their regulation by neurotransmitters and intracellular messengers. We are now addressing these problems by combining the tools of electrophysiology and of nucleic acid molecular biology. The major preparation is the *Xenopus* oocyte; RNA encoding various membrane channels is microinjected into this cell. Many of the studies are conducted in collaboration with the group of Prof. Norman Davidson in the Division of Chemistry and Chemical Engineering.

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221. **Mouse-Torpedo Hybrid Acetylcholine Receptors: Functional Homology Does Not Equal Sequence Homology**

Michael M. White, Katharine Mixter Mayne, Henry A. Lester, Norman Davidson

The nicotinic acetylcholine receptor (AChR) is a
multisubunit protein complex of stoichiometry a2βγδ. The several subunits show homology with each other within a given species; in addition, homology is found between analogous subunits between species. We have used the phage SP6 RNA polymerase transcription system to produce single-species RNA in vitro for various AChR subunits from cDNAs. Injection of an equimolar mixture of RNA for the α, β, γ, and δ subunits of the Torpedo californica AChR into Xenopus oocytes results in the appearance of functional receptors in the oocyte membrane. No response to acetylcholine (ACh) is detected when the β or γ subunit RNA is omitted, and a small response is seen when the δ subunit RNA is omitted. Replacement of the Torpedo δ subunit RNA by the mouse BC3H-1 cell line AChR δ subunit RNA leads to the formation of functional receptors that show a 3–4 fold greater response to ACh than does the full Torpedo complex. No response is seen when the mouse δ RNA replaces Torpedo γ RNA. By amino acid homology profile comparisons, the mouse δ subunit appears to be moderately but not highly similar to the Torpedo δ subunit; the apparent similarity to the Torpedo γ subunit is only slightly less. Therefore, the features of the primary sequence that determine the functional δ character of the mouse polypeptide are not revealed by simple homology comparisons.

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222. Big and Little Patches on Xenopus Oocytes

Henry A. Lester, Nathan Dascal, Michael M. White

With the help of R. Nuccitelli, B. Sakmann, and B. Gillo, we have developed methods to obtain gigohm seals with these cells. The procedure involves a 2-hr exposure to collagenase to remove the follicle cells, followed by a 15-min exposure to hypertonic solution; this treatment causes the plasma membrane to shrink away from the vitelline membrane, and the latter is then removed with fine forceps.

The naked plasma membrane seems to have remarkable sealing properties. Using standard fire-polished patch pipettes, we obtain better than 80% success at seals of resistance >10^10 ohms. Single channels are readily resolved with such patches. In most cases, these patches can be converted to stable outside-out excised patches by the standard mechanical manipulations. In about 20 outside-out patches, however, we have not yet recorded single ACh-induced channels after injection of RNA coding for the ACh receptor. We hypothesize that the receptors are clustered on the membrane, so that random patches will show no channels. We are therefore experimenting with various mapping procedures for identifying the most promising regions to obtain gigohm seals.

The successful seals also led to a new technique for measuring macroscopic currents from membrane areas several hundred µm² in area and with a temporal resolution (~200 µs) sufficient for detailed kinetic studies on voltage-dependent conductances. The method involves a fire-polished pipette with a tip diameter of 20–50 µm. This is pushed against the oocyte cell membrane; gentle suction is applied and the membrane within the patch is drawn into the tip of the pipette. Within a few min a gigohm seal also forms. The enclosed membrane area typically contains several hundred or several thousand channels.

223. Microinjection of RNA into Xenopus Oocytes as a Tool for Molecular Cloning of a Rat Brain Serotonin Receptor Gene

Hermann Lübbert, Nathan Dascal, Terry P. Snutch, Henry A. Lester, Norman Davidson

The low abundance of most voltage and/or neurotransmitter-sensitive receptors and ion channels renders it difficult to prepare pure proteins and obtain either amino acid sequence data or antibodies. Since for nearly all channels and receptors these useful tools for molecular cloning are unavailable, we envisioned a cloning strategy based on the observation of Barnard et al. (1982) that a number of voltage-sensitive or neurotransmitter-responsive channels appear on the surface membrane of Xenopus oocytes after microinjection of rat brain poly(A)+ RNA.

We propose to use this as a selection system for the cloning of a rat brain serotonin (5-HT) receptor gene. This approach also has aided in the cloning of other genes for which activity tests are applicable. The oocyte membrane contains an endogenous muscarinic acetylcholine (ACh) receptor which is coupled to a chloride channel (Kusano et al., 1977; Dascal et al., 1984). It has been shown that after injection of rat brain poly(A)+ RNA into Xenopus oocytes, a rat brain 5-HT receptor is also coupled to a chloride channel...
channels activated by agonist (GABA, kainate, serotonin) and voltage (Na, K, Cl) in oocyte's membrane. Two to three days after injection of brain, muscle, and heart RNA, an additional depolarization-activated current was resolved with 40 mM external Ba, 0 external Cl, and intracellular TEA injections. This current was blocked by 0.1 mM Cd or 2 mM Co but not by TTX. The peak amplitude was as high as 250 nA and correlated well with that of the Ca-activated Cl current observed in the same cells in normal physiological solution. We conclude that the Ba current flows through voltage-dependent calcium channels (VDCs).

The first step in our intended cloning procedure was to size fractionate rat brain poly(A)+ RNA by electrophoretic separation through agarose gels. A single ~6 kb RNA fraction, enriched at least 60 fold, was sufficient to induce a serotonin response in D2 components. We have constructed cDNA clones of this ~6 kb RNA fraction and are working on a selection procedure for positive clones.

References:

1Division of Chemistry and Chemical Engineering, California Institute of Technology.

224. Voltage-dependent Calcium Channels in Xenopus Oocytes Injected with Exogenous mRNA

Nathan Dascal, Terry P. Snutch1, Hermann Lübbert1, Norman Davidson1, Henry A. Lester

As reported earlier by other groups (Gundersen et al., 1983; Houamed et al., 1984), the injection of mRNA from brain resulted in the appearance of ion channels activated by agonist (GABA, kainate, serotonin) and voltage (Na, K, Cl) in oocyte's membrane. Two to three days after injection of brain, muscle, and heart RNA, an additional depolarization-activated current was resolved with 40 mM external Ba, 0 external Cl, and intracellular TEA injections. This current was blocked by 0.1 mM Cd or 2 mM Co but not by TTX. The peak amplitude was as high as 250 nA and correlated well with that of the Ca-activated Cl current observed in the same cells in normal physiological solution. We conclude that the Ba current flows through voltage-dependent calcium channels (VDCs).

The Ba currents from 8-day RNAs peak at +10 mV and show a 30-50% decline during a 1-sec depolarization; 14-day brain RNA induces Ba currents with a peak at 0 mV and half-decay times of about 70-80 msec. Ba currents in heart, muscle, and brain mRNA-injected oocytes were substantially enhanced and prolonged by exposure to the adenylate cyclase activator, forskolin. Similar enhancement was observed for several minutes after direct intracellular pressure injection of cyclic AMP; this effect was repeated several times in a given oocyte.

By comparing results with total, poly(A)+, and poly(A)+ mRNAs, we conclude that the calcium channels are synthesized from poly(A)+ mRNA. RNA was injected from rats of various ages; the synthesis of heart and muscle VDCs is maximal between six and eight days postnatal.

References:

1Division of Chemistry and Chemical Engineering, California Institute of Technology.

225. Rat Brain Serotonin Receptor May Couple to Endogenous Inositol Second Messenger System in Xenopus Oocytes

Arie M. Michelsohn

Acetylcholine binds at muscarinic receptors on the membrane of the Xenopus oocyte and evokes an electrical response with a characteristic shape and time course. The two significant features of this response appear to be a phase that occurs within 5 sec after agonist application, and another that begins to appear after approximately 1 min. Both phases consist of an outward chloride current which is calcium dependent.

Upon injection with poly(A)+ RNA from rat brain, the oocyte becomes physiologically responsive to serotonin. This response has the characteristic features of the oocyte's endogenous response to acetylcholine; further, ACh and serotonin apparently display mutual occlusion and cross desensitization. It has been reported that muscarinic stimulation of the oocyte induces phosphatidylinositol (PI) turnover, thus liberating the second messenger inositol triphosphate, a molecule known to release calcium from internal
stores. Our experiments are aimed at demonstrating a link between the biochemical induction of PI turnover and the onset of the physiological response to serotonin, thus substantiating our hypothesis that the response to the two agonists is indeed identical. Our biochemical assay measures the accumulation of inositol phosphate after timed exposures to either ACh or serotonin. Experiments are performed on single oocytes, whose physiological responses can be monitored and correlated with the biochemical data. Preliminary results suggest that serotonin promotes a 40% increase in inositol phosphate levels within 30 sec of stimulation with the agonist.

226. Acetylcholine and Phorbol Esters Inhibit Potassium Currents Evoked by Adenosine and Cyclic Adenosine Monophosphate in Xenopus Oocytes

Nathan Dascal, Ilana Lotan 1, Boaz Gillo 1, Henry A. Lester, Yoram Loss 1

In Xenopus laevis oocytes, adenosine and other purinergic agonists induce a K+ conductance increase which is fully mimicked by intracellular application of cyclic adenosine monophosphate (cAMP). Acetylcholine (ACh) suppresses the potassium conductance increase caused by adenosine, by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), or by intracellular injection of cyclic AMP (cAMP). This effect of AChCho is not mimicked by intracellular injection of calcium ions or of the Ca-mobilizing agent, inositol 1,4,5-trisphosphate (Ins 1,4,5P3). However, adenosine and cAMP responses are inhibited by the phorbol esters 4α-phorbol-12,13-dibutyrate (PBt2) and 4β-phorbol 12-myristate 13-acetate (PMA). These results suggest that in Xenopus oocytes, the muscarinic inhibition of purinergic and cAMP responses is mediated through the activation of the phospholipid-dependent, Ca-activated protein kinase (protein kinase C).

1 Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Israel.

227. Effects of Phorbol Esters on the Heart

Alison M. Gurney, Nathan Dascal

The sympathetic and parasympathetic nervous systems have antagonistic effects on cardiac function. The increased force and rate of contraction of the heart, produced by sympathetic nerve stimulation or α-adrenergic agonists, arise primarily from an enhanced slow inward current (Iα) carried mainly by calcium ions. These effects are opposed by the parasympathetic transmitter, acetylcholine (ACh), which decreases Iα and enhances a time-independent outward potassium current (IK), through activation of muscarinic receptors. Although it is now clear that activation of cardiac α-adrenoceptors enhances Iα through stimulation of the enzyme, adenylate cyclase, and elevation of intracellular levels of cyclic AMP (cAMP), it is still not known what mediates the effects of ACh.

Muscarinic receptors were recently shown to be linked to the turnover of phosphatidyl inositol (PI) in cardiac membranes. Hydrolysis of PI in the plasma membrane yields, among other things, diacylglycerol, which activates protein kinase C. Phorbol esters, which act like diacylglycerol, and protein kinase C have profound effects on voltage-dependent Ca and K currents in some tissues. Phorbol esters were also shown recently to inhibit the response to intracellular injection of cAMP in Xenopus oocytes. As yet, however, no direct link has been established between this putative messenger and heart cell conductance. Thus, its role in mediating muscarinic effects in heart is not clear.

We tested the effects of the phorbol esters, phorbol dibutyrate (PBt2) and phorbol myristate acetate (PMA) on the rate and force of contraction of isolated cardiac preparations from guinea pigs and frogs. Atria were dissected free from guinea pig hearts, and short lengths of cotton thread attached to the extreme tips of each atrium. One piece secured the atria to a hook at the bottom of a perfused organ bath, while the other piece was attached to an isometric transducer positioned above the bath. Whole frog hearts were mounted in a similar manner. The transducer monitored the tension developed during each heartbeat. PBt2 (1 µM) caused a small reduction in the force and rate of contraction, within a few minutes, and inhibited the response to 0.2 µM isoprenaline by around 15% (six experiments) in guinea pig atria. Similar effects were observed in the isolated frog heart. PMA (10 µM), by itself, reduced tension in the frog heart by 20-30% and antagonized the response to isoprenaline. Although preliminary, these data do support a role for protein kinase C in modulating the
effects of muscarinic agonists on the heart. Only small drug effects were expected as the experiments provided a rather insensitive assay. We hope to determine the significance of these results by improving the sensitivity in future experiments. We plan to study the effects of phorbol esters and protein kinase C directly on \( I_{\text{si}} \) and \( I_K \) in isolated heart cells, using "patch clamp" recording techniques.

228. Effects of Dihydropyridine Ca\(^{2+}\) Antagonists in Bag Cell Neurons of Aplysia californica

Jeanne M. Nerbonne, Alison M. Gurney

Brain tissue is rich in high affinity binding sites for dihydropyridine Ca\(^{2+}\) antagonists and may prove useful for isolating and purifying neuronal Ca\(^{2+}\) channels. It must first be clearly determined, however, that these binding sites have a physiological correlate and represent a uniform class of Ca\(^{2+}\) channels. We have characterized the effects of the antagonists, nifedipine and nisoldipine, on neuronal Ca\(^{2+}\) currents (\( I_{\text{Ca}} \)), and compared the efficacy and selectivity of current suppression to previous observations in cardiac muscle.

Voltage-activated transmembrane currents were studied, using the "whole-cell" mode of patch-clamp recording, in a variety of dissociated neuronal cells maintained in culture. \( I_{\text{Ca}} \) recorded from rat superior cervical ganglion, rat dorsal root ganglion or chick ciliary ganglion neurons was unaffected by both antagonists at concentrations to 10 \( \mu \)M. These drugs did, however, suppress the amplitude of \( I_{\text{Ca}} \) recorded from the neurosecretory bag cells of the marine mollusc Aplysia californica. Inhibition by nifedipine was half maximal at 1.7 ± 0.2 \( \mu \)M, compared with 300 \( \mu \)M required for 50\% suppression of \( I_{\text{Ca}} \) amplitude in cardiac cells. Similar mechanisms may underlie the blockade in the two cell types. As in heart cells, nifedipine and nisoldipine appeared to interact with the Ca\(^{2+}\) channel in at least three states. Kinetic studies suggest that the drugs interact with resting, closed channels, open and inactivated Ca\(^{2+}\) channels. Thus, as in cardiac muscle, the effects of dihydropyridine antagonists on \( I_{\text{Ca}} \) in bag cells are explained by the "modulated receptor" model of drug action, which proposes that the affinity of the drug for its receptor is modulated by the state of the channel. These Ca\(^{2+}\) antagonists were not, however, selective for \( I_{\text{Ca}} \). Nifedipine and nisoldipine also suppressed two outward K\(^{+}\) currents, identified as the "delayed rectifier" and the "A" current. Concentrations as low as 3-5 \( \mu \)M nifedipine were sufficient to cause 50\% inhibition of either current. Kinetic studies revealed several similarities between the effects of these drugs on K\(^{+}\) currents and \( I_{\text{Ca}} \) and the effects on K\(^{+}\) currents were similarly explained by the "modulated receptor" model. Interestingly, the dihydropyridine Ca\(^{2+}\) agonist, BAY K 8644, was without effect on either current (at 1-10 \( \mu \)M), but protected against the inhibitory effects of the antagonists on both Ca\(^{2+}\) and K\(^{+}\) currents.

In bag cells, as in heart, the affinity of nifedipine was highest for inactivated Ca\(^{2+}\) channels, with a \( K_D \) = 350 nM. This value is much higher than dissociation constants measured in binding studies on neuronal tissue. Thus, it seems unlikely that both studies reveal the same binding sites. In view of this, and the lack of selectivity of the dihydropyridines in bag cells, more information is clearly needed on the physiological effects of dihydropyridine Ca\(^{2+}\) antagonists on vertebrate brain neurones.

229. Development of Voltage-activated Conductances in Sympathetic Neurons

Jeanne M. Nerbonne, Alison M. Gurney, Helen B. Rayburn

The whole-cell patch clamp recording technique has been employed to study the development of electrically excitable membrane properties in superior cervical ganglion (SCG) neurons in dissociated culture. SCG cells have been isolated from neonatal and embryonic (E14.5-19.5) rats by enzymatic digestion and grown in monolayer cultures in MEM supplemented with 10\% horse serum and NGF. Within 2-4 hours after plating, cells adhered to collagen-coated glass coverslips and whole-cell recordings could be obtained.

With 140 mM Na\(^{+}\) and 5-10 mM Ca\(^{2+}\) in the bath and 140 mM K\(^{+}\) in the pipettes, depolarizations from holding potentials (hps) -30 to -80 mV revealed fast inward TTX-sensitive Na\(^{+}\) (\( I_{\text{Na}} \)) and delayed, outward K\(^{+}\) (\( I_K \)) currents in voltage-clamped neonatal SCG cell. In the absence of \( I_{\text{Na}} \) (2 \( \mu \)M TTX), depolarizations from hps -70 to -90 mV also activated a transient outward K\(^{+}\) current (\( I_A \)) in addition to \( I_K \). Replacement of the K\(^{+}\) with Cs\(^{+}\) (140 mM) in the pipettes blocked outward currents and revealed the presence of a slow
inward current blocked by Co²⁺ and carried by Ca²⁺ (I_{Ca}). These currents were present in SCG cells within hours of isolation and we assume, therefore, that they are present in vivo. In addition, the properties of the recorded currents did not change over several weeks in culture. Our results are, therefore, qualitatively similar to those reported (Freschi, 1983), on six-week (in culture) neonatal SCGs and, more recently on adult cells (Belluzzi et al., 1985). Although the same currents were observed, some qualitative differences between our results and the previous studies were apparent. These differences appear merely to reflect the more complete current separation achieved using the whole-cell recording technique.

Similar experiments have also been completed on embryonic (El4.5-19.5) SCGs in dissociated cultures. We have found that cells from late (≥E17.5) embryos possess the full complement of voltage-activated currents with the same properties as those seen in neonatal cells. Early embryonic (El4.5-15.5) cells, however, did not display the fast transient outward current Iₐ; I_{Na}, I_{K} and I_{Ca} were all present and their properties indistinguishable from those seen in cells from neonates and late embryos. When these El4.5-15.5 cells were maintained in culture, Iₐ was seen to develop over the next several days (after plating) and, at 6 days, Iₐ was found in all cells examined. Iₐ develops, therefore, in vitro over approximately the same time course as it seems to be developing in vivo.

Although no precise function has yet been ascribed to Iₐ sympathetic neurons, it is possible that it is responsible for fast repolarization of the action potential. If this is the case, then, the development of Iₐ likely reflects a developmentally relevant change in the shape of the action potential and in the overall excitability of SCG cells.

References:

230. Photochemically Generated Concentration Jumps of Cyclic GMP in Bullfrog Rod Photoreceptors

Alison M. Gurney, Grant Nicol

When illuminated, vertebrate rod photoreceptors hyperpolarize as a result of the closing of cation-selective ion channels in the cell membrane. The coupling of the photopigment, rhodopsin, to changes in membrane conductance is known to involve an intracellular second messenger, although the nature of this messenger(s), and how it modulates conductance, is unknown. One possible candidate is cyclic GMP (cGMP). Strong support for this was provided in recent studies, which showed that cGMP increases conductance in isolated patches from the membrane of rod outer segments. It has been proposed that cGMP, present in the rod cell cytoplasm, holds membrane cationic channels open in the dark. On exposure to light, the bleaching of rhodopsin ultimately results in an increased activity of phosphodiesterase, which hydrolyzes cGMP to GMP, resulting in channel closure. We aimed to test this hypothesis by producing intracellular concentration jumps of cGMP in bullfrog rod photoreceptors. This was made possible by the recently synthesized analogue of cGMP, the 4,5-dimethoxy-2-nitrobenzyl ester of cGMP. Irradiation of this ester, at wavelengths of 280-420 nm, results in liberation of free cyclic GMP; this is complete in less than 5 msec. Thus this molecule is, in effect, a "caged" cGMP.

Photoreceptors were bathed in physiological solutions containing "caged" cGMP at concentrations to 500 μM, in the absence and presence of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX; 100 μM). The rod inner segment was drawn into the tip of a large pipette such that a seal (−10 MΩ) was formed between the pipette tip and the junction of inner and outer segments. The pipette was used to monitor the current flowing from the rod outer segment into the inner segment, and thus the current flowing through the light-sensitive channels. Cell viability was checked by delivering low intensity light flashes of wavelengths >500 nm; slow current decreases of around 20 pA, as seen in the absence of drug, were usually observed, indicating that the light response was unaltered by the presence of "caged" cGMP. High intensity, wide-spectrum light flashes of 1 msec duration, which converted 5-10% of the "caged" cGMP, were then delivered. We expected to see a fast increase in current, as the photogenerated rise in cGMP caused channels to open, followed by a slower decrease, as cGMP was hydrolyzed and channels closed. However, no clear effect was observed in the
absence of IBMX, and in the presence of IBMX, an unexpected result was obtained. As was anticipated, the response to the long wavelength flash was slowed by IBMX. In contrast, however, the response to the high intensity flash was very much faster in the presence of IBMX. The reasons for this are still unclear. It may be caused by the change in concentration of photoproducts other than cGMP, or perhaps the mechanisms by which these light-sensitive channels are modulated are more complex than presently believed.

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231. Ca$^{2+}$-activated K$^+$ Currents Studied With Photochemically-generated Intracellular Jumps in Ca$^{2+}$ Concentration

Alison M. Gurney

Ca$^{2+}$-activated K$^+$ channels have mainly been studied in isolated patches of cell membrane, since this allows the Ca$^{2+}$ concentration on the intracellular side of the membrane to be more readily controlled. In intact cells, Ca$^{2+}$-sensitive currents are not readily isolated from other voltage-activated currents. If inward Ca$^{2+}$ currents are blocked, by application of inorganic or organic channel blockers, then the Ca$^{2+}$-sensitive currents, which are activated by Ca$^{2+}$ entering the cell through Ca$^{2+}$ channels, are also blocked. Similarly, pharmacological blockade of other outward K$^+$ currents usually results in inhibition of Ca$^{2+}$-activated K$^+$ currents. The synthesis of a new photo-sensitive Ca$^{2+}$ chelate, nitr-2, by Roger Tsien at Berkeley, now permits the study of Ca$^{2+}$-activated currents in intact cells. This molecule, which is a derivative of the Ca$^{2+}$ chelate, BAPTA, is membrane impermeable and binds Ca$^{2+}$ with a K$_D$ of 0.17 µM. Irradiation at wavelengths 280-350 nm results in a molecule that binds Ca$^{2+}$ with a K$_D$ of 7 µM. Thus, a light flash at these wavelengths causes an increase in the concentration of free Ca$^{2+}$, the magnitude of this change depending on the intensity of the light.

The "whole-cell" mode of patch clamping was used to record currents in dissociated superior cervical ganglion cells, in which Ca$^{2+}$-activated K$^+$ currents have previously been identified. Recordings were made in physiological saline containing low concentrations of Ca$^{2+}$ (1 mM); 1 mM CdCl was sometimes added to block residual Ca$^{2+}$ currents. Nitr-2 (2 mM) was introduced into the cells, along with Ca$^{2+}$ buffered to nominally 1 or 10 nM, by addition to the K$^+$ solution in the patch pipette. After obtaining a gigaohm seal between the pipette and the cell membrane, suction applied through the pipette ruptured the membrane patch, allowing the cell to be voltage clamped and the nitr-2 to dialyze into the cell. Flashes presented when the cell was clamped at potentials positive to -40 mV resulted in an outward K$^+$ current that rose with a time constant of 150-200 msec. It is not yet clear what process this time constant reflects. Unfortunately, the release of Ca$^{2+}$ by nitr-2 does not occur instantly, rather the time constant for release in solution is 450 msec. It is hoped that, in the future, better compounds will be developed that release Ca$^{2+}$ more rapidly. The amplitude of the current was larger at more positive potentials. The relationship between current amplitude and the membrane potential was similar to previous findings with single-channel recordings of Ca$^{2+}$-activated K$^+$ channels.

The results thus far suggest that light-sensitive Ca$^{2+}$ chelates such as nitr-2 may prove useful in studying Ca$^{2+}$-dependent processes inside cells. A detailed study of the kinetics of these processes must wait, however, until the synthesis of new and improved molecules that will provide step changes in intracellular Ca$^{2+}$ concentration.

232. Involvements of G Proteins in Serotonin Response in Xenopus Oocytes Injected with Brain mRNA

Nathan Dascal, Catherine K. Ifune, Rosemary Hopkins, Terry P. Snutch, Melvin I. Simon, Henry A. Lester

Xenopus oocytes injected with rat brain poly(A)$^+$ mRNA develop serotonin (5-HT) and acetylcholine (ACh) responses. The 5-HT and ACh responses in these oocytes are similar in shape and both are dependent on internal calcium. Exposing the oocytes to 5-HT first and then to ACh, or vice versa, shows that there is cross-desensitization between the two responses, indicating that these responses are expressed through the same pathway. We believe that the release of calcium from the internal stores of the oocyte is linked to phosphoinositide metabolism. There is evidence that guanine nucleotide binding regulatory proteins (G proteins) are involved in phosphoinositide turnover regulation. Since it is known that pertussis
Toxin ADP-ribosylates the inhibitory G protein of adenylate cyclase, we have studied the effect of pertussis toxin on 5-HT and ACh responses on the oocytes.

Treatment of mRNA-injected oocytes with pertussis toxin reduced the 5-HT and ACh responses by 50% compared to nontreated cells. This decrease was specific for 5-HT and ACh since there was little or no effect of pertussis toxin on other responses such as GABA and kainite. We propose that the decrease is due to the inhibition of phosphoinositide hydrolysis by pertussis toxin.

The injection of the γ-β subunits of the G protein involved in photoreception (transducin) has been found to cause a twofold enhancement of the 5-HT response. Again, there was little or no effect on other responses. It should be mentioned that the γ-β subunits of transducin are identical to γ-β subunits of other G proteins.

We conclude that there are G proteins similar to those that regulate adenylate cyclase that couple serotonin and ACh receptors to phosphoinositide turnover. Next we are planning to further study this mechanism, e.g., by observing the effects of guanine nucleotides and cholera toxin.

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233. Microcircuit Culture Dishes
John R. Gilbert, Becky G. Tanamachi, Brian Rasnow, Jerome Pine

Development has continued on the construction of tissue culture dishes that incorporate an array of 61 extracellular stimulation and recording electrodes. The durability of the insulating layer has been sufficiently improved so that initial experiments can begin.

The first experiments will be study of synapse elimination at neuromuscular junctions formed in cocultures of chick ciliary neurons and chick skeletal muscle. By utilizing the microcircuit electrodes, selected neurons can be stimulated so as to establish chronic activity patterns and study their effect on nerve-muscle synapses. For example, muscles will be identified that are doubly innervated, and the effect of activity imposed on one input on the relative synaptic strengths will be determined. At present, the optimal conditions for cocultures are being developed, and the dishes needed for the experiments are being made.

The nerve-muscle experiments will utilize intracellular recordings in the muscle cells to detect the connectivity and to study relative synaptic strengths, with the dish electrodes utilized solely for stimulation. In other planned experiments described below, the microcircuit dishes will be used to study neural networks, and optical recording will be used to measure neural activity and relative synaptic strengths. For these experiments, microcircuit dishes with transparent electrodes formed with indium tin oxide are now being developed.

234. Development and Plasticity of Small Neural Networks
Helen B. Rayburn, Chi-Bin Chien, Brian Rasnow, Becky G. Tanamachi, Jerome Pine

Planned experiments involve the study of microcultures of approximately ten neurons grown over a microcircuit electrode array, which will permit controlled stimulation of the individual cells. A voltage-sensitive fluorescent dye, used to stain the cell membrane, will provide the means for measuring the membrane voltage changes associated with synaptic inputs and action potentials.

By measuring the stimulus-response properties of the network at a given time, the electrophysiological connectivity of the culture can be observed. Afterward, chronic stimuli will be applied by driving appropriate microcircuit electrodes, utilizing small microprocessor-based controllers. The change in the stimulus-response properties of the culture with time will reveal effect of electrical activity on synaptic connectivity, provided controls are maintained for effects of normal development. The non-invasive techniques, which have now been developed for stimulation and recording, will allow such measurements to be made for the first time.

During the past year a microculture system was developed and the first dye recordings were made that show the response of each cell in the culture to a stimulus applied to a single neurite bundle. The stimulus for these tests was an extracellular micropipette, simulating the action of a microcircuit electrode incorporated into the culture dish. In order to perform the planned experiments, microcultures will be grown on 61-electrode microcircuit culture
dishes, so that stimulation of any individual cell of the culture can be achieved. A multidetector optical recording system is now being designed that will yield simultaneous recordings of the response of the entire culture network to a given stimulus. A microcircuit dish with transparent conductors is also being developed to facilitate optical recording.

During the year, detailed tests were conducted to optimize the conditions for dye recording, including studies of the effects of the culture medium, the duration of staining, and the dye concentration. Other tests were made to compare the usefulness of over 20 different styryl dyes kindly supplied by Prof. Amiram Grinvald of the Weizmann Institute. From these tests, two or three dyes are established as best suited for our purposes. In addition, some insights into possible structural correlates with dye performance are suggested. In the coming year, we will collaborate with Angelika Arnold-Mittelstein, a student in Prof. Sunney Chan's group in the Division of Chemistry and Chemical Engineering, in studies of the biophysical basis of the dye action.

235. A Neuron-Silicon Connection

Wade Regehr, Jerome Pine

During the past year we have begun a study directed toward the development of a new technology for making chronic electrical connections between neurons and external electronic circuitry. The basic concept is to fabricate in silicon and silicon dioxide a microdevice that forms a cup-shaped contact over part of the membrane area of a cultured cell, not unlike the contact made with a patch electrode for a whole-cell recording. The silicon structure can be formed by using newly developed anisotropic etching techniques. Within the cup and insulated from the silicon with oxide, a gold electrode will be formed that will be connected to external electronics for stimulation or recording. Prof. David Rutledge of the Division of Engineering and Applied Science is working with us on this fabrication project and providing the facilities of the Caltech integrated circuits lab.

In order to test the basic concept, structures that we call "diving board electrodes" are being fabricated for making contact with cultured neurons. The electrode has a pedestal that will be attached to the culture dish bottom, and from which projects a long flexible arm, about 200 microns long, one micron thick, and ten microns wide (the diving board). Within the arm, an insulated conductor will make contact with an electrode in a cup formed at the end of the board. The structure will be micromanipulated into position, so as to establish contact between the cup and a cultured neuron, and the pedestal will then be cemented to the bottom of the culture dish. Tests will be made of the mechanical and electrical properties of this system, and of the response of neurons to the close chronic contact with the cup structure.

It is hoped that with proper surface treatment of the cup electrode, a high resistance contact will be made with the neural membrane. Given such a "gigohm seal" it will then be possible to electrically puncture the cell membrane within the cup and to obtain a chronic intracellular connection. If the initial efforts are successful, a variety of culture experiments will be facilitated. Furthermore, the same basic technique can be applied to the production of implantable probes containing "tame neurons," which can interact with the nervous systems of intact organisms. The long-range potential of such probes includes studies of the biological function as well as neural prostheses to compensate for some forms of impaired neural function in humans.

236. Soft X-Ray Microscopy

Jerome Pine, Deanna Deeds

The first scanning transmission soft x-ray microscope to be built in this country is being developed at the National Synchrotron Light Source by a team of physicists from the State University of New York, the IBM Watson Labs, and the Light Source. We have been participating in ongoing tests to evaluate the instrument as it is being perfected. The microscope should ultimately make possible the imaging of live cultured neurons at a resolution of about 50 nanometers. This is of particular interest to us as a possible tool for directly observing synaptic structures.

The basic principle of the microscope is to utilize x-rays with a wavelength of about 3 nanometers, which are weakly absorbed by water and strongly absorbed by carbon. As a result, natural contrast for observing the proteins and lipids of living cells is obtained without staining. A significant additional advantage of using x-rays is that since they are uncharged, they do not multiply scatter in thick specimens with resultant loss
of resolution. The main problem in building the microscope is to image the incident x-ray beam as a small spot, whose diameter determines the resolution of the device. This is done with a zone plate, whose construction requires state of the art microfabrication. At present the resolution of the microscope is about 200 nanometers, as defined by the zone plate properties, which is competitive with light microscopy at its best.

During the summer of 1984 we took pictures of fixed wet cultures, and attained the limiting resolution described above. The microscope pictures are obtained in the form of x-ray counts for each pixel of the image. These data were processed at Caltech on the Astronomy computer system, using image processing software modified for our purposes from that used by the astronomers. Thin neurites were better resolved than in phase contrast, but it was not yet possible to observe synaptic structures or intracellular organelles. During the coming year we expect to obtain pictures at higher resolution, and we will also begin to use live cells.

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PUBLICATIONS


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Professor: Paul H. Patterson
Research Fellows: William V. Bleirsch, Arlene Y.-C. Chiu, Keiko Fukada, Irene V. Pech, Randall N. Pittman
Special Graduate Student: Josette F. Carnahan
Research Staff: Joann Imrich, Doreen McDowell, Marie Ryder

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Summary: The interests of this laboratory concern two aspects of the development of the nervous system: the factors controlling which type of phenotype a neuron acquires, and identification of molecules involved in intercellular interactions leading to synapse formation. Much of our effort has centered on sympathetic neurons developing in cell culture. These neurons can become adrenergic or cholinergic, and a glycoprotein secreted by heart cells can control this transmitter choice without affecting neuronal growth or survival. This protein has recently been purified and is being sequenced by Drs. Ruedi Aebersold and Stephen Kent in Prof. Lee Hood's group. Future work will involve cloning the gene for this differentiation signal and studies of the control of its expression. The role of hormones and growth factors has been extended to other phenotypic choices in this cell lineage, and interconversions of adrenal medullary cells, SIF (small intensely fluorescent) cells and neurons have been demonstrated in culture. The control of these interconversions is now being studied in vivo using antibody injections and hormone perturbations. Thus, this area of work involves the identification, purification and study of the molecular action of cues in the embryonic environment that influence phenotypic choices in various lineages of the developing nervous system.

The extensive synaptic connections elaborated by neurons in culture provide an assay system for identifying neuronal macromolecules—surface-bound or secreted—which mediate intercellular interactions. A number of surface glycoproteins are found only on neurons, and one of these is modified and released into the medium when the neurons are stimulated to undergo exo- and endocytosis. The structure of this protein and its possible functional roles are under study. Recent results have implicated a protease and its irreversible inhibitor in interactions with neuronal surfaces and the extracellular matrix. These interactions have been further shown to control axon outgrowth and regeneration. The interaction of neurons, target cells, and glia via such molecules is being studied using immunological tools. A novel method for producing monoclonal antibodies against rare antigens was devised, and an antibody that directly blocks the activity of a neurite outgrowth-promoting complex was produced. This antibody is being used to localize the outgrowth activity in situ. Thus, this second area of work involves the identification of molecules involved in intercellular interactions leading to axon outgrowth, regeneration and synapse formation.

237. In situ Localization of a Neurite Outgrowth-promoting Factor in Rats

Arlene Y.-C. Chiu, Michael DeFreitas1, Josette F. Carnahan, Paul H. Patterson

During development and regeneration, neurons extend processes over long distances before reaching target sites. A variety of non-neuronal cells, including muscle and glia, secrete a factor that will adhere to surfaces and induce neurons to rapidly extend processes in vitro (Lander et al., 1982). Neurite-promoting factors from a number of different sources are found to be made up of a complex of two molecules—laminin (LMN) and a heparin sulfate proteoglycan (HeSPG)—which are commonly found in the extracellular matrix and on cell surfaces. Antibodies directed against each of these components can precipitate the complex out of solution but are unable to directly block its neurite-promoting activity. However, a novel immunological procedure produced monoclonal antibodies that inhibit neurite outgrowth (INO), presumably recognizing an epitope close to the active site (Matthew and Patterson, 1983). Interestingly, the INO antibody binds to the factor only when LMN and HeSPG are in association.

We have looked at sites of INO binding in the rat peripheral and central nervous systems and compared them to the distribution of LMN and HeSPG using several monoclonal antibodies. INO, LMN and HeSPG immunoreactivity co-localize to a great degree. In the peripheral nervous system, all three are present on surfaces of Schwann cells in peripheral ganglia as well as along peripheral nerves and dorsal and ventral roots. In contrast, no immunoreactivity is seen on surfaces of cells and nerves of the central nervous system. This differential distribution is intriguing in light of the finding that grafts of peripheral, but not central, nerves will support regeneration of both peripheral and central neurons (Benfey and Aquayo, 1982). Skeletal and cardiac muscles, as well as muscle spindles, are also immunoreactive for INO, LMN and HeSPG. However, INO staining is absent at the neuromuscular junction where motoneurons stop growth to form a nerve terminal. These results are consistent with
biochemical evidence that the INO epitope requires a LMN-HeSPG complex. We also find that the extracellular matrix in non-neuronal tissue, such as kidney and blood vessels, contain LMN and HeSPG but little INO immunoreactivity. It will now be important to examine the distribution of INO during development when neurons first extend neurites.

References:
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238. Proteases, Their Inhibitors, The Extracellular Matrix and Neurite Outgrowth
Randall N. Pittman, Paul H. Patterson

What are the molecular mechanisms that allow neuronal axons to grow invasively for long distances and yet stop at the appropriate locations? Recent results suggest that proteases and their inhibitors may be involved in neurite outgrowth and interactions with the extracellular matrix. Sensory and sympathetic neurons spontaneously release a collagenase and a plasminogen activator from their distal processes and/or growth cones (Pittman, 1985). A 43 kd irreversible inhibitor of the plasminogen activator is secreted by cardiac myocytes and is found on the surface of cultured neurons (Pittman, 1984). This inhibitor is also released by cultures of peripheral, but not central, nerves. This is of interest because the components of peripheral, but not central, nerves can support the regeneration of both peripheral and central axons. Furthermore, Monard et al. (1983) have found that this 43 kd inhibitor can stimulate neurite extension from neuroblastoma cells. Of interest in relation to the neurite-promoting proteoglycan complex described in the preceding report is the preliminary finding that the 43 kd inhibitor binds heparin sulfate (HeS) tightly and can displace laminin from its HeS binding site. This result raises the possibility that the protease/inhibitor system could affect outgrowth via interaction with the neurite-promoting complex in the extracellular matrix.

References:

239. Purification of a Glycoprotein that Controls the Transmitter Phenotype of Developing Neurons
Keiko Fukada

The existence of a diffusible factor that controls the choice of transmitter in developing sympathetic neurons, without affecting neuronal survival or growth, has been demonstrated in culture (Patterson, 1978). In order to understand the molecular mechanism of its action and its role in development, purification of the molecule is essential.

Using conventional biochemical techniques, the protein has been purified >10^5 fold with a reasonable recovery from serum-free, hormone-supplemented, heart cell conditioned medium (Fukada, 1983). The most purified fraction is active at <10 ng/ml and retains the ability to both inhibit the development of noradrenergic characteristics as well as to induce cholinergic differentiation. The activity has the same apparent molecular weight of 45 kd under denaturing and native conditions, suggesting that the factor is a single subunit. The 45 kd area where activity resides on SDS gels is not iodinated by methods that label tyrosine residues but is well labeled by the 125I-Bolton-Hunter reagent, which labels lysine residues. The 45 kd protein is very likely to be the cholinergic factor because of the following results. 1) The activity and the iodinated 45 kd protein are both sensitive to trypsin but not to chymotrypsin, and the 45 kd protein is only labeled by Bolton-Hunter iodination. 2) When the iodinated Sephadex fraction is analyzed by two-dimensional gel electrophoresis, each of the 125I-labeled spots precisely lines up with the activity peaks in the second dimension. 3) Removal of carbohydrate from the labeled 45 kd protein with endoglycosidase F gives a single band of 22 kd, and the cholinergic activity comigrates with this 22 kd protein. Thus, the cholinergic factor is a slightly basic molecule whose core protein is composed of a single polypeptide of 22 kd, and the biological activity resides in the protein moiety (Fukada, 1985). N-terminal sequencing of this molecule is currently in progress in collaboration with Drs. Ruedi Aebersold and Stephen Kent.

References:
240. Production of Monoclonal Antibodies against SIF Cells

Irene V. Pech

Small intensely fluorescent (SIF) cells are a minority population of cells in sympathetic ganglia. SIF cells share some differentiated characteristics in common with sympathetic neurons and adrenal medullary cells, and arise from the same embryonic source, the neural crest. It has been postulated that SIF cells may be the precursor cells for the entire sympathoadrenal lineage (Landis and Patterson, 1981). One way to test this idea would be to inject antibodies that recognize SIF cells, but not neurons, into developing rats. If complement-mediated death of SIF cells also results in a deficit in the neuronal and chromaffin cell populations, the hypothesis that SIF cells are progenitors would be greatly strengthened.

To obtain such antibodies, mice were injected with a homogenate of newborn rat sympathetic ganglia and immunosuppressed with cyclophosphamide (Matthew and Patterson, 1983). This injection procedure was repeated until complete suppression of the response to ganglion cells had occurred. Then, in vitro immunization with neonatal rat carotid body membranes was performed. The carotid body, located at the bifurcation of the carotid artery, contains cells which are very similar to SIF cells and is a more plentiful source of tissue to use as an immunogen than SIF cells themselves.

Screening of the resulting clones eliminated antibodies that stained muscle and/or sympathetic neurons and glial cells. The remaining supernatants are being tested for their ability to stain frozen sections of newborn rat carotid body. Those that are positive will then be used to again screen sympathetic ganglion sections. Serial sections will be cut and alternate sections will be processed for Falck-Hillarp fluorescence to ensure specificity of the antibodies for SIF cells. Such antibodies can then be injected into developing rats and their effects determined.

References:

241. Control of Developmental Phenotypic Interconversions in vivo

Josette F. Carnahan

Adrenal chromaffin cells, sympathetic neurons and small intensely fluorescent (SIF) cells are all neural crest derivatives and show a number of features in common, but they can be distinguished by morphological, biochemical and immunological criteria. Since cultured SIF cells can be converted into adrenal chromaffin cells or neurons by manipulation of corticosteroid and nerve growth factor (NGF) levels (Doupe et al., 1985), it has been suggested that SIF cells may give rise to these other cell types during normal development. This hypothesis is being tested by observing the consequences of altering the number of SIF cells during development by treating rat embryos with anti-NGF and glucocorticoids. The first step of this project has been to purify to homogeneity of the β subunit of NGF from mouse submaxillary glands in order to prepare a clean antiserum to NGF.

Reference:

242. Structure and Function of a Neuronal Surface Protein

William v. Bleisch

In an effort to determine the role of surface proteins in neuronal development and function, we have described two surface proteins of sympathetic neurons that are released from the cell surface during or following depolarization (Sweadner, 1983). This suggests that these proteins could be messengers, carrying information to other cells or to other parts of the same neuron. A monoclonal antibody that recognizes one of these proteins was produced and the protein was shown to be the NILE protein, which is found on the surfaces of most, if not all, neurons and neuronal cell lines. The other released protein, B2, behaves like NILE in all respects but is not immunologically related, and unlike NILE, has so far only been found on the surface of sympathetic neurons. We have begun studies of the structure and function of these two proteins. In particular, we are continuing the study of the mechanisms for release of these proteins. In addition, we are using the antibody to purify enough of the NILE protein to begin studies of the structure of
this molecule and to characterize the structural changes that precede release of this protein from the cell surface. We also are comparing NILE with other surface proteins, particularly the family of neural cell adhesion molecules. We are also attempting to produce additional monoclonal and polyclonal antibodies that recognize NILE as well as the related B2 protein. Towards this goal, we are attempting to improve techniques for production of monoclonal antibodies against surface antigens that are specific to particular cell types, by combining various techniques to select for a specific population of spleen cells before spleen-myeloma fusion.

References:

PUBLICATIONS


Summary: Our laboratory uses a combination of methodologies—including classical and molecular genetics, electrophysiology, and neuroanatomy—to study the nervous system of the fruitfly, Drosophila melanogaster. Our main interest is in those mechanisms that generate cellular diversity within the nervous system. At present, our major focus is on neural excitation. The tonic bias of neural excitation is well understood. Membrane molecules act as channels allowing ions to enter or leave the cell. Different channels are selective for different ionic species; temporal sequences of their openings and closings generate the action potential. Action potentials are not identical in all nerve cell types; some neurons fire with single action potentials, others fire in bursts or act as pacemakers. Differential distributions of channels apparently account for these differences in membrane excitability.

We are examining the genetics and molecular biology of two major, voltage-activated, ionic channel classes in Drosophila: 1) the Na+ channel, and 2) the A channel, a K+-selective channel. Mutations in several Drosophila loci are known to affect Na+ channels. Although these loci interact genetically, the genetics remain unclear because it is not known which locus encodes the channel protein. The abstract by Mani Ramaswami describes our attempts to identify this locus.

Assistant Professor: Mark A. Tanouye
Research Fellow: Linda E. Iverson
Graduate Students: Alexander Karnb, Mani Ramaswami
Laboratory Staff: Ross J. McMahon

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Physiological studies in several laboratories have identified different classes of K⁺-selective channel. All classes are selective for K⁺ but differ in features such as channel activation requirements (e.g., voltage-sensitive activation and Ca**⁺-dependent activation), kinetics of activation and inactivation, and pharmacological sensitivities. Different combinations of K⁺ channels may be the basis for many of the differences in excitability properties among different neurons. In addition, some evidence suggests that simple forms of learning and memory are mediated by modulation of K⁺ channel activity.

Despite their importance, virtually nothing is known about K⁺ channel molecules. This is primarily due to technical difficulties associated with biochemical purification of these channels. We have used a strategy that combines classical and molecular genetics to identify the genes that encode or regulate the expression of K⁺ channels. Previous work has shown that Shaker (Sh) mutations in Drosophila eliminate or modify one class or K⁺ channel, the A channel. The Sh locus has been mapped cytologically using a collection of Sh translocation stocks to within a few bands at 16F on the X chromosome. Several EMS-induced Sh alleles have been mapped genetically with respect to each other, and with respect to the translocation breakpoints. Sh appears to be a complex genetic locus. In the abstracts by Alexander Kamb and Linda Iverson, we report the molecular cloning and partial characterization of the region containing Sh.

**243. Molecular Biology of Sh**

Linda E. Iverson, Alexander Kamb

We obtained genomic DNA clones of the Sh complex using a cDNA clone (adm 135 H4, isolated by M. Wolfner) that maps by in situ hybridization to 16F on the X chromosome. We have used phage libraries constructed in our laboratory as well as the "Maniatis" lambda library to extend the cloned region in both directions. Recently, we constructed a cosmider library that we are using to "skip" along the chromosome. To date, over 175 kb of DNA has been cloned from the Sh complex. Currently, we are searching for the translocation breakpoints which flank the Sh.

We have characterized a portion of the cloned DNA in more detail. One restriction fragment that includes an entire transcription unit has been transferred into flies via P element-mediated embryo injection. This transferred gene complements the recessive lethal mutation, **t**₂. The **t**₂ gene is one of three recessive lethal complementation groups that maps immediately (-0.03 cm) distal to the dominant Sh mutations such as **Sh**KS¹³³. The function of the **t**₂ gene is unknown, but genetic mosaic studies indicate that it affects nerve and/or muscle.

**244. Isolation of Sh Revertants**

Alexander Kamb, Linda E. Iverson

We have begun to examine some of the more bizarre aspects of Sh genetics. First, we generated flies carrying a homozygous deletion of the Sh complex. This deletion includes the recessive lethal complementation groups **t**₁, **t**₂ and **t**₃. This puzzling result has been labeled "impossible" by the world's leading geneticists.

Based on the results described above, it should be possible to obtain revertants of **t**₁, **t**₂ and **t**₃. We are in the process of mutagenizing **t**₁-₃ stocks with X rays to look for such revertants.

All previous attempts to isolate Sh⁺ revertants from Sh strains have failed. The major obstacle to these efforts has been the difficulty of identifying wild-type flies against a background of Sh mutants. To circumvent this problem, we are constructing an ether-a-go-go (eag), Sh double mutant. Sh, eag flies have downturned wings. By screening for normal wings, it should be possible to identify any Sh⁺ revertants. Sh revertants may be useful in two ways: 1) they may aid in localizing the site of Sh dominant mutations such as **Sh**KS¹³³; and 2) "second site" revertants may identify genes whose products interact with Sh.

**245. Cloning of the Na⁺ Channel Gene in Drosophila**

Mani Ramaswami

Several mutants with altered sodium channel activity are known in Drosophila. The major alleles map to three loci: no-action-potential (nap¹⁸), seizure (set¹⁸), and para¹⁸. All three are temperature-sensitive paralytics that rapidly undergo reversible paralysis at their restrictive temperatures. There are strong genetic interactions between these mutant groups but it is not clear which, if any, of them codes for the structural protein of the voltage-sensitive sodium channel.
Recently, sodium channel genes from two systems have been cloned. The Norman Davidson group in the Division of Chemistry and Chemical Engineering at Caltech has used an immunological screening of a λgt11 library to obtain sodium channel clones from the rat. These show considerable homology with the cloned channels from the electric organ of the eel *Electrophorus electricus* (Noda et al., 1981).

Using the mammalian cDNA clones from A. Goldin and A. Dowset of the N. Davidson lab, we are attempting to clone the sodium channel gene in *Drosophila*. We are at present adjusting conditions to obtain unique signals with heterologous probes on a Southern blot. Screening of a *Drosophila* genomic library followed by *in situ* hybridizations to salivary gland chromosome should determine if any of the known membrane-excitability mutants correspond to the structural Na+ channel protein.

References:

246. Genetic Analysis of *Bendless*

**Susannah J. Hannaford**, Katherine Uchida, Alexander Kamb

The giant fiber pathway mediates the jump/flight escape response in *Drosophila*. Mutations that interrupt this pathway, such as *Bendless* (*ben*), render mutant flies unable to jump. *ben* is believed to affect one neural connection in the pathway. Thus, *ben* may encode a molecule that directs the formation of a specific synapse. Only one *ben* mutant allele has been isolated (Thomas and Wyman, 1982). We are mutating flies with EMS, X rays, and hybrid dysgenesis (transposon insertion mutagenesis) to isolate additional *ben* mutants. Dysgenesis-induced mutant alleles may be used to obtain DNA clones of the *ben* locus in order to characterize the structure and function of the *ben* gene product.

References:

**Professor Emeritus**: Anthonie Van Harreveld

**Support**: The work described in the following research report has been supported by:
Divisional Funds
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247. Comparison of the Features of Two Types of Retinal Spreading Depression

**Anthonie Van Harreveld**

Two types of spreading depression (SD) have been demonstrated in the isolated chick retina. One form is suppressed by 10 mM MgCl₂ in the bathing solution (Mg-sensitive SD). The other type can be elicited in retinas bathed in a salt solution containing 10 mM MgCl₂ and 10-20 mM KC! (Mg-insensitive SD). In addition to the difference in Mg sensitivity, the two types show differences in other features, which are summarized in Table 1. The Ca²⁺ requirements of the two types of SD are quite different. The Mg-insensitive SD needs at least 1 mM CaCl₂ in the medium, whereas Mg-sensitive SD can be elicited in a bathing solution containing as little as 0.1 mM CaCl₂. The rate of propagation of the Mg-sensitive SD decreases markedly with the decline of the Ca concentration (3.7 mm/min in 1.5 mM CaCl₂, 0.6 mm/min in 0.1 mM CaCl₂). Both forms of SD are suppressed by Co²⁺ and Ni²⁺, which suggests the involvement of Ca²⁺-mediated processes. This is supported by the possibility of replacing Ca²⁺ by Ba²⁺ in the Mg-insensitive SD. Remarkable is the need of a high KC! concentration (10-20 mM) in the medium supporting the Mg-insensitive SD. The suppression of the Mg-insensitive SD by 10 mM TEA suggests that in
this type of SD a K⁺ current is an essential feature of its mechanism. Neither of the two types of SD is affected by TTX, indicating that no voltage-dependent Na⁺ channels are involved. The considerable differences in the features of the two types of SD strongly suggest that different mechanisms are involved in their propagation.

PUBLICATIONS


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**Table 1**

*Features of Two Types of Spreading Depression*

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mg-insensitive SD</th>
<th>Mg-sensitive SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg sensitivity</td>
<td>Not suppressed by 10 mM MgCl₂</td>
<td>Suppressed by 10 mM MgCl₂</td>
</tr>
<tr>
<td>CaCl₂ requirement</td>
<td>At least 1.0 mM</td>
<td>0.1 mM suffices</td>
</tr>
<tr>
<td>Rate of propagation</td>
<td>2.24 ± 0.10 mm/min (n = 12)</td>
<td>3.68 ± 0.12 mm/min (n = 12)</td>
</tr>
<tr>
<td></td>
<td>28°, 1.5 mM CaCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.60 ± 0.04 mm/min (n = 15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28°, 0.1 mM CaCl₂</td>
<td></td>
</tr>
<tr>
<td>Effect of Co²⁺ and Ni²⁺ or NiCl₂</td>
<td>Suppressed by 2 mM CoCl₂ or 4 mM NiCl₂</td>
<td>Suppressed by 2 mM CoCl₂</td>
</tr>
<tr>
<td>Replacement of Ca²⁺ by Ba²⁺</td>
<td>1.5 mM CaCl₂, can be effect of Ba²⁺</td>
<td>SD is prevented by cytotoxic</td>
</tr>
<tr>
<td>replaced by 2 mM BaCl₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl requirement</td>
<td>10-20 mM is required</td>
<td>1-3 mM suffices</td>
</tr>
<tr>
<td>Effect of tetraethylammonium</td>
<td>Suppresses SD in 10 mM</td>
<td>Not affected by 10-15 mM TEA</td>
</tr>
<tr>
<td>concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrodotoxin sensitivity</td>
<td>Not affected by 1 µM TTX</td>
<td>Not affected by 1 µM TTX</td>
</tr>
</tbody>
</table>
Summary: This year we have begun to explore the organization of the primate visual system with histochemical and immunocytochemical methods. We have found monoclonal antibodies that appear to be specific to fiber systems connecting to the Middle Temporal Visual Area (MT) with other cortical areas (see Abstract No. 249). We have also used histochemical methods to investigate the organization of the visual system in rare primates, such as tarsiers and lemurs, that have died from natural causes in breeding colonies at Duke University. These studies have revealed a striking correlation between nocturnality and the pattern of acetylcholinesterase staining in the dorsal lateral geniculate nucleus of the thalamus (see Abstract Nos. 248 and 250).

Laminar Distribution of Acetylcholinesterase and Cytochrome Oxidase in the Dorsal Lateral Geniculate Nucleus of *Lemur fulvus*

Colin T. McDonald, EveLynn McGuinness, John M. Allman

We examined the dorsal lateral geniculate nucleus (DLGN) of the adult and infant *Lemur fulvus albifrons*, a prosimian native to Madagascar. The DLGN contains six cell layers: two ventral magnocellular (MC) layers and two dorsal parvocellular (PC) layers separated by two lighter staining koniocellular layers. In cytochrome oxidase-reacted sections of both the adult and infant lemur, dense staining is seen in both the MC and PC layers, but not the two koniocellular layers. Staining is most intense in the anterior region of both the external PC layer and the external MC layer, which correspond to the peripheral visual field representation.

Acetylcholinesterase (AChE) reaction product is densest in the two PC layers of the adult lemur. This result closely resembles the findings in nocturnal primates: the tarsier (E. McGuinness, personal observation) and the owl monkey and bushbaby (Fitzpatrick and Diamond, 1980). In contrast, the DLGN of the diurnal macaque monkey (Manocha and Shantha, 1970), squirrel monkey (Hess and Rockland, 1833) and marmoset (personal observation) show densest AChE staining in the MC layers with only light staining in the PC layers. Within the external PC layer of adult lemur, the densest AChE staining is in the peripheral field representation. In contrast to the adult, the two koniocellular layers of the infant lemur show densest AChE staining and the reaction product is located in cell bodies.

*Lemur fulvus* has usually been considered to be diurnal, but recent studies indicate that the *albifrons* subspecies is active at night as well and has a round-the-clock or die! activity pattern (Conley, 1975; Tattersal, 1982). Thus they must use their visual system in conditions of low illumination like nocturnal primates.

We would like to thank the Duke University Primate Center for the Study of Primate Biology and History for providing us fresh frozen tissue from lemurs that had died of natural causes.

References:
Because antigens that define extrastriate visual areas might be present in minute quantities in the primate brain, a modification of the immuno-suppression protocol of Matthew and Patterson (1983) was employed. Mice were initially immunized with marmoset whole brain homogenate from a non-visual area (frontal cortex, excluding frontal eye fields). The immunosuppressant drug cyclophosphamide was then introduced to suppress the mouse immune system. The mice were given a final boost of marmoset whole brain MT homogenate without suppression. In this way, we hoped to minimize the production of monoclonal antibodies against common antigens, and therefore for this experiment, uninteresting primate brain antigens.

Antibody 2E10, one of the six specific extrastriate markers, labels in the adult owl monkey a discrete bundle of axons ventral to the MT region and below cortical layer VI. Label is seen projecting dorsally and anteriorly toward the superior temporal sulcus and on to parietal cortex. Of developmental interest is the fact that this antibody shows a slightly more diffuse pattern of staining in the newborn marmoset, labeling a larger area of visual cortex.

References:

250. Organization of the Visual System in Tarsiers
EveLynn McGuinness, John M. Allman

In primates the dorsal lateral geniculate nucleus (DLGN) is the principal relay of visual input from the retina to the cerebral cortex. We have investigated the organization of the DLGN in coronal and parasagittal sections of the brain of a *Tarsius bancanus* and a *Tarsius syrichta*. Alternate serial sections of the *T. bancanus* were stained for cell bodies and for fibers. In the *T. syrichta* we stained four series of sections for cell bodies, fibers, acetylcholinesterase, and cytochrome oxidase. The DLGN contains two ventral magnocellular layers, a broad interlaminar zone and two dorsal parvocellular layers, which is the basic pattern found in all other haplorhine primates; all strepsirhines possess these layers plus two additional layers containing very small cells which are intercalated between the two dorsal layers (Kaas et al., 1978). The organization of the DLGN in the tarsier is the simplest of any primate and most closely resembles the DLGN of the owl monkey. The organization present in the DLGN in strepsirhines appears to have been derived from a pattern like that present in the tarsier but contains two additional specialized laminae. In addition we found in the acetylcholinesterase-stained sections a pattern that is literally as clear as night and day correlates with the tarsier's nocturnality. In the tarsier the two dorsal parvocellular layers are densely stained for acetylcholinesterase, the two ventral magnocellular layers are lightly stained. In the nocturnal owl monkey and galago, the parvocellular layers are densely stained (Fitzpatrick and Diamond, 1980). In diurnal macaque monkey (Manocha and Shantha, 1970; Graybiel and Ragsdale, 1980), squirrel monkey (Hess and Rockland, 1983) and marmoset (personal observations), the ventral layers are more densely stained, which is the opposite of the pattern found in the three nocturnal primates that have been investigated.

Fitzpatrick and Diamond (1980) demonstrated in galagos that the dense parvocellular staining was not reduced by eliminating the retinal input. Similarly, kainic acid injections into the DLGN, which produced severe cellular destruction, did not reduce acetylcholinesterase staining in the parvocellular laminae. However, Fitzpatrick and Diamond (1980) found that primary visual cortex lesions eliminated the dense staining in the visuotopically corresponding parts of the parvocellular laminae. They also found acetylcholinesterase-positive neurons in layer VI of primary visual cortex, which is known to project back to the DLGN. These results strongly suggest that layer VI of primary visual cortex is the source of the acetylcholinesterase-rich input to the parvocellular laminae in galagos. By contrast, Hess and Rockland (1983) found no evidence of a similar defect in the acetylcholinesterase staining of the magnocellular laminae of diurnal primates (macaque and squirrel monkeys) following lesions in primary visual cortex.

Taken together, these results suggest that in nocturnal primates there is an acetylcholinesterase-rich projection feeding back from the primary visual cortex to the parvocellular laminae of the DLGN that is not present in diurnal primates. The presence of
acetylcholinesterase does not necessarily indicate that the acetylcholine is the neurotransmitter in the cortico-parvocellular feedback system. It has been suggested that acetylcholinesterase may regulate or amplify the effects of other transmitter systems (Brzin et al., 1982). It is conceivable that acetylcholinesterase is serving to enhance in nocturnal primates the responses of the neurons in the parvocellular laminae, which are much less sensitive to low contrast stimuli than are neurons in the magnocellular laminae.

We thank Drs. Elwyn Simons and Patricia Wright of the Duke University Center for the Study of Primate Biology and History for providing the brains of tarsiers that have died of natural causes in their breeding colony.

References:

251. Organization of Owl Monkey Extrastriate Cortex

Martin I. Sereno, John M. Allman

It has been known for some time that there are a large number of small, extensively interconnected, more or less topographic maps of the visual field beyond the large primary visual cortex (V-I) in primates. However, roughly half of the areas have not been explored thoroughly enough to unambiguously establish their singleness, internal visuotopic organization (if any), and borders with surrounding areas, and almost nothing is known about differences between areas at the level of the dendritic and axonal morphology of single neurons.

We have therefore resumed fine-grained mapping experiments in the owl monkey. Previous mapping experiments and quantitative studies concentrated on five extrastriate areas—V-11, MT, DL, DM, and M. There remained a considerable region of visual cortex rostral, medial, and ventral to these areas that has been less well explored. Preliminary results suggest that there are at least two dorsoventrally elongated areas rostral to MT characterized by large to very large receptive fields. In spite of this, receptive field progressions show a degree of visuotopic order. There also appears to be another visuotopically organized area containing lower and upper visual fields just lateral to the rostral tip of the ventral (i.e., upper visual field) wing of DL in caudal inferior temporal cortex. This last area is rostral to the ventral strip-like areas VA and VL.

A second project is to examine the morphology of selected "feedforward" projections at the single axon level. Partly because it is difficult to obtain Golgi-like HRP labeling of neuronal processes beyond about 5 mm away from an extracellular or intracellular injection site, almost nothing is known about the detailed morphology of interareal corticocortical axons. We have begun trial injections in rat visual cortex using the lectin PHA-L as an anterograde marker since it produces Golgi-like staining at greater distances. Eventually, we plan to reconstruct single interareal axon terminals in owl monkey DL and caudal inferior temporal cortex after PHA-L injections into V-11 and DL, respectively. With double injections, we should be able to directly assess the degree of convergence at the single axon level.

252. Central Nervous System Electrophysiology in a Visually Predatory Arachnid

David W. Stivertsen

Jumping spiders (salticidae) are visual predators. These arachnids do not capture prey with silk webs but rely on visually guided behaviors. Brightly colored salticids wave their front legs like semaphores as a courtship display and stalk their prey with relentless efficiency, dropping into a cat-like crouch to stalk prey beyond the reach of their leap. The Caltech campus is home to several species, including Phidippus johnsonii, a large black, red, and iridescent green spider.

Previous work has quantified behavior (Land, 1972; Forster, 1982), receptor properties and anatomy (Hill, 1975). Ethological studies relating brain and behavior have been hampered by technical difficulties in recording signals from the central nervous system. I have developed techniques that open this area to study.
Salticids have eight eyes. One pair is largely vestigial, two pair are primarily concerned with orienting toward visual stimuli, and the anterior medial (AM) pair perform complex visual analysis, similar to foveal vision in mammals. The AM eyes have 2° wide by 20° high retina. The receptors are ordered into four distinct layers, which are tiered along the 2° width. This array along the optical axis takes advantage of the chromatic aberration to focus green and UV wavelengths independently.

Although the lenses of the principal eyes are fixed in the chitinous exoskeleton, the retinas are mobile. Land (1969) has shown that salticids can scan the visual field of the lenses with a variety of stereotyped retinal movement patterns, similar to vertebrates. The use of a narrow high resolution retina to raster a large visual field is an elegant solution to the compromises inherent in a miniature and compact system.

Using computerized stimulus presentation and data collection techniques adapted from primate research, I have characterized receptive fields and their properties. There are similarities to vertebrates, such as cells that are extremely velocity selective. In addition, I have found two novel properties with single multi-unit recording in the CNS. The first is receptive fields which are "space-mapped." These cells respond to stimuli in a constant spatial region that is independent of the retinal map, with fields larger than the width of the retina.

The second novel property is monocular depth perception. Many visual receptive fields in these spiders and in vertebrates subend a constant angle, independent of stimulus distance. In P. johnsonii, I have found "size-constant" cells. The angular subtense of the receptive field diminishes with distance, with the absolute physical size remaining constant. This type of processing requires depth information. I believe this information is extracted using the tiering of the retinal layers. Translational eye movements result in receptors being present at different distances from the focal point along the optical axis. For near field vision, objects at different distances have different points of best focus.

Future plans include cobalt injections for anatomical tracing of connections involved in these processes.

References:

Publications

Assistant Professor: James M. Bower
Laboratory Staff: David H. Bilitch, Joan L. Roach

Support: The work described in the following research reports has been supported by:
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The National Institutes of Health, USPHS

Summary: The overall objective of research in our laboratory is to understand how information is processed by neuronal circuitry in cortical regions of the mammalian brain. To understand the underlying
principles of this information processing, two separate neural structures are studied—the piriform (cerebral) cortex and the cerebellar cortex. Both of these structures have a regular stereotyped structure that lends itself well to physiological analysis, are laminary organized, and have extensive intrinsic fiber systems mediating interactions between neurons. These two cortical structures differ markedly, however, in the temporal and spatial patterns of activity evoked in them by peripheral stimuli (Bower and Woolston, 1983; Haberly and Bower, 1984). The objectives of this research group are first to understand in each case the physiological characteristics of the circuitry underlying these different activity patterns, and then, using this knowledge, to explore the significance of these characteristics for the function of each structure.

Three very different, but complementary experimental approaches are employed to describe these two cortical systems. Questions about the basic physiological organization of these neural networks are addressed using in vitro brain slice techniques, where the basic physiology of cortical neurons can be analyzed, and the influences of single neurons on other neurons within a network can be studied directly (Bower and Haberly, 1984). This preparation is also used with anatomical techniques that reveal the morphological features of individual neurons and the spatial distributions of specific axonal systems within each cortex. The in vitro preparation is also being used in conjunction with immunocytochemical techniques (in collaboration with Profs. Mary Kennedy and Paul Patterson) to study the biochemistry of each cortex.

The second experimental approach being developed involves the use of large arrays of recording elements to simultaneously monitor the responses to peripheral stimuli of many individual neurons in intact (in vivo) subjects (Bower and Llinas, 1983). These "multi-unit" experiments will employ extracellular recording techniques that rely heavily on computer-based data acquisition, analysis, and display procedures for their interpretation.

The third experimental approach to be taken in this lab involves building realistic computer models of each cortex. These models are based on the structural and physiological features determined using the in vitro preparations, and will be evaluated on their accuracy in predicting the results of the in vivo multi-unit experiments. Thus, the models serve as the unifying focus for the research efforts of this group. The models will also provide a means of applying information gained about activity in local neural circuits to the understanding of information processing in much larger arrays of cortical neurons.

References:

253. Facilitating and Nonfacilitating Synapses on Pyramidal Cells: A Correlation Between Physiology and Morphology

James M. Bower, Lewis B. Haberly

The main objective of these in vitro brain slice experiments was to compare the excitatory post-synaptic potentials (EPSPs) generated in olfactory cortical pyramidal cells by two different fiber systems—the afferent system arising in the olfactory bulb, and the intrinsic association system arising from pyramidal cells within the cortex itself. Our results revealed a marked difference in physiology between the synapses, which is correlated with a difference in synaptic morphology. Specifically, we found that EPSPs evoked by primary olfactory tract afferents that terminate on distal apical dendritic segments of pyramidal cells display paired shock facilitation (i.e., the second of two identical stimuli produces a much larger EPSP), while EPSPs evoked by intrinsic association fibers that terminate on proximal apical segments do not facilitate. Further, the ultrastructural comparison of the presynaptic elements of these two fiber systems showed that the facilitating olfactory tract afferent synapses have a much lower packing density of synaptic vesicles than do the nonfacilitating association fiber synapses. A subsequent search of the literature revealed that where both morphological and physiological data are available, this same correlation appears to apply for a wide range of other excitatory synapses. Based on these experiments we have proposed a hypothesis to account for this general correlation, which is based on
the ability of synaptic vesicles to buffer internal calcium. Aspects of this hypothesis are currently being pursued using immunocytochemical techniques in collaboration with Prof. Mary Kennedy. While these data may have provided insight into the mechanism underlying synaptic facilitation, the descriptions of the differences between EPSPs evoked by these two fiber systems are also important to our efforts to model this cortex.

Associate Professor, Department of Anatomy, University of Wisconsin, Madison, WI.

254. Multi-single Unit Recording in Cerebellar Cortex: Developing a New Technical Approach

James M. Bower

An abundance of morphological and physiological data concerning the structural organization of the central nervous system suggest that its neural networks process information in an intrinsically parallel fashion. This is clearly evident in the many circuits that transmit the same bits of afferent sensory information to different brain structures for simultaneous processing. Further, and of particular relevance to our experimental work, parallel computational schemes are also employed in the neural networks that comprise many, if not all, brain structures. While it is clear that the central nervous system relies heavily on parallel processing, experimental methods that allow the direct sampling of the activities of many individual functionally-related neurons are as yet largely undeveloped. The overall technical objective of this project, therefore, is to apply modern microfabrication techniques to the design and construction of micro-electrodes, which will allow the activities of many individual neurons in cerebellar or cerebral cortex to be monitored simultaneously. This experimental approach will also require the development of new computer algorithms for multi-channel data acquisition and analysis. Specifically, these projects involve the development of: 1) multi-ported, silicon-based electrodes using metal etching or metal deposition techniques; 2) a very precisely engineered device, probably also made of silicon, to hold several of these electrodes in a fixed configuration for insertion into the brain; 3) the design and manufacture of miniaturized multi-channel preamplifiers and processing amplifiers; and 4) the development and implementation of new algorithms based on information theory for deciphering and analyzing neuronal activity in real time. Installation of the computer, which will be central to this undertaking, has been accomplished, and the development of the data handling software is under way. In addition, initial design of the necessary microdevices has begun.

PUBLICATION


Professor: Masakazu Konishi
Research Fellows: Catherine E. Carr, Daniel Margoliash, Winthrop E. Sullivan, Terry T. Takahashi, Susan Volman, Hermann F. Wagner
Graduate Student: Richard D. Mooney
Member of the Professional Staff: Eugene Akutagawa
Laboratory Staff: Charlene Huang

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Summary: During the past year we have made a number of significant observations in our studies of the owl's auditory system and the song control system of birds. We wanted to know how the barn owl measures interaural time differences in the microsecond range. We have reason to believe that the third order nucleus in the owl's auditory system contains coincidence detectors inserted in asymmetrical delay lines. We also have elucidated how the owl's auditory system derives interaural time differences from interaural phase differences.

One of the brain areas that controls song contains neurons sensitive to sound. A study of this area in white-crowned sparrows shows that the most effective stimulus is the individual bird's own song. Because the sparrow must hear himself sing in order to develop song, the song selectivity of the area must be due to vocal-auditory interaction during song development. The song control areas show marked sexual di-
morphism, particularly in species like the zebra finch in which the female does not sing at all. Brain sexual dimorphism was thought to originate from the differential growth of the relevant areas. We now know that neuronal growth in the male and atrophy and death in the female account for the sexual dimorphism of the song system in the zebra finch.

255. Parallel Pathways in the Owl's Brainstem Auditory System

Terry T. Takahashi, Mark Konishi

Barn owls localize sounds by deriving interaural time and intensity differences. Evidence from single unit recordings suggests that time and intensity are processed in independent neural channels ascending, respectively, from nucleus magnocellularis (NM) and nucleus angularis (NA), the avian cochlear nuclei. We report here the projections of the cochlear nuclei and nucleus laminaris (NL) as determined by 3H-proline and horseradish peroxidase (HRP) injections. Nucleus laminaris was studied because our evidence suggested that it is the sole target of the projections of NM. Proline injections were confined to NA in three owls. Collectively, their loci and sizes were such as to involve all of NA. In every case, label was observed bilaterally in the superior olive (SO) and nucleus lemnisci lateralis pars ventralis and contralaterally in caudal inferior colliculus (IC), nucleus ventralis lemnisci lateralis pars posterior (VLVp), and rostral pole of nucleus lemnisci lateralis pars anterior (VLVa). Injections of HRP into each of these labeled areas produced retrograde-labeled cells in NA, suggesting that they receive direct projections from NA. Proline injections confined to NM (N = 2) labeled only NL of both sides. HRP injections into nuclei other than NL failed to produce retrograde-labeled cells in NL. These observations suggest that NM projects only to NL. These projections of NL were studied in six owls, one of which received three equally-spaced proline injections along the rostro-caudal nuclear axis. The remainder received only one injection. Virtually all parts of NL were involved. The injection was confined to NL in one bird (single injection); in others, part of NM was also involved. In all cases, label appeared over the ipsilateral SO and the contralateral VLVa and rostromedial IC. No additional areas were labeled in cases in which the injections encroached upon NM. HRP injections into the labeled regions produced retrograde-labeled cells in NM, suggesting direct projections. The pathway from NM to the rostromedial IC and VLVa (via NL) may form the anatomical substrates of the time channel and the pathway from NA to caudal IC and VLVp may form that of the intensity channel.

256. Construction of a Neural Map of Interaural Phase Difference in Nucleus Laminaris of the Barn Owl

Winthrop E. Sullivan, Mark Konishi

We studied the mechanisms underlying neuronal sensitivity to interaural phase difference in the barn owl's nucleus laminaris, the first-order binaural nucleus in the avian brainstem. Using micro-electrodes, we recorded a large sinusoidal response to tonal stimuli, or "neurophonic" response, in this nucleus. The neurophonic has a 2 to 3 msec latency and is sharply tuned to frequency. These and other properties show its physiological origin. The neurophonic is probably due to the synchronized, phase-locked activity of a population of neurons. In the owl, neurophonic potentials can be recorded at frequencies of up to 8.5 kHz, and can be driven by monaural excitation of either ear or by binaural stimulation. As the electrode moved ventrally within the nucleus, the neurophonic response to a contralateral stimulus showed a progressive phase advance whereas a phase delay was seen in successive ipsilateral ear responses. Binaural responses were sensitive to interaural time delay, a maximum response occurring when the monaural responses would be in phase and a minimum response occurring when they would be out of phase. Binaural tuning curves shifted towards a greater ipsilateral time lead with depth.

The results are consistent with the bilateral innervation pattern of nucleus laminaris by the cochlear nuclei, and support a simple model for the genesis of binaural phase tuning and a neural map of interaural phase difference. Ipsilateral cochlear nucleus fibers enter nucleus laminaris from the dorsal side and contralateral fibers enter ventrally. The electrode moves towards the contralateral and away from the ipsilateral source as it advances ventrally, thus causing an earlier contralateral and a later ipsilateral response.
In our model, fibers from the left and right magnocellular nuclei project through nucleus laminaris in opposite directions, representing the opposing "delay lines" proposed by Jeffress (1948). This generates a dorsal-ventral gradient of neural delay difference. For coincidence of monaural signals to occur, increases in neural delay must be balanced by decreases in acoustic delay. Thus, a ventral movement causes binaural neurophonic tuning curves to shift towards a smaller acoustic delay in the ear for which a longer neural delay is seen, i.e., the ipsilateral ear. Laminar neurons also respond maximally when the two monaural inputs coincide. This "coincidence detection" transforms the neural delay difference gradient into a map of interaural phase (or time) difference.

Reference:

257. Organization of Nucleus Laminaris in the Barn Owl

Catherine E. Carr, Nicholas C. Brecha¹, Mark Konishi

Sensitivity to interaural temporal disparities underlies aspects of sound localization. The first place where this sensitivity appears is nucleus laminaris (NL), which is also the first recipient of binaural input. An analysis of the structure and connections of NL has been undertaken to identify morphological correlates of the emergent sensitivity to interaural temporal disparities. The Golgi technique, immunocytochemical techniques and in vivo and in vitro intracellular dye injection were used.

In the barn owl, NL is a greatly hypertrophied brainstem nucleus, surrounded by afferent fiber tracts. NL contains numerous large (30-50 µm) neurons in a central neuropil area, and these cells have many short stubby dendrites, from 10 to 50 µm in length. NL neurons receive afferent input from both the ipsilateral and contralateral cochlear nucleus magnocellularis. Nucleus magnocellularis receives eighth nerve input via the end bulbs of Held directly onto the unipolar somata of its neurons. Each neuron projects bilaterally to nucleus laminaris, and their terminals within NL are large, stubby and have few branches. These terminals end closely apposed to the large cells of NL. NL also contains a small cell type of unknown function.

Immunocytochemical methods have revealed that Calcium Binding Protein (CaBP) immunoreactivity is localized to both the neurons of nucleus magnocellularis and NL, and the target of the NL afferents, VLVa (anterior part of the ventral nucleus of the lateral lemniscus), which is also sensitive to interaural temporal disparities. Glutamic acid decarboxylase (GAD) immunoreactivity has demonstrated the presence of a second afferent input to NL: GAD-positive terminals surround the somata of the large cells of NL. They are of unknown origin, and their presence suggests that inhibitory processes may act in NL.

Sensitivity to a particular temporal disparity is conferred by the simultaneous arrival of afferent magnocellular inputs onto the cells of NL. The time difference in the incoming signals is compensated for within NL (Sullivan and Konishi, 1985). Physiological and ultrastructural techniques will be used to analyze how the requisite temporal advances and delays are produced.

Reference:

¹CURE, Wadsworth VA, Westwood, Los Angeles, CA.

258. Derivation of Characteristic Delay

Terry T. Takahashi, Mark Konishi

The auditory system of the barn owl derives the characteristic delay (CD) from the interaural phase difference of each frequency in a signal. We report a neural parallel of this computational process in the space-specific neuron of the owl's inferior colliculus. When stimulated with a tone, the space-specific neuron's discharge is a cyclic function of interaural delay, which has a period equal to that of the stimulus tone. Changing the stimulus frequency changes the periodicity of the delay-response functions, but one interaural time difference (ITD) evokes a maximum response, regardless of frequency. This ITD is the CD. When stimulated with noise, the response is again cyclic with a period equal to that of the unit's best frequency, but the response at the CD is, on average, twice as strong as the response to ITDs that are one period removed. Thus, the space-specific neuron is able to unambiguously signal the CD when provided...
with a signal having a wide spectrum. This is a result of a facilitation of the unit's response to the CD and a suppression of the response to the ITD that are one period removed from the CD. The suppression and facilitation observed with noise stimuli were also observed when the summed waveform of the best frequency and another tone, F2, were presented. The response of the space-specific neuron to these two-tone stimuli could not be accounted for by the summing or averaging of the delay-response functions obtained with the best frequency or F2 alone, suggesting that non-linear neural processes are involved.

259. Minimal Signal Duration for Sound Localization in the Barn Owl

Hermann F. Wagner, Mark Konishi

The barn owl can localize the azimuthal position of a target very precisely by using ongoing interaural time differences in signals containing frequencies between 5 and 9 kHz. Neurons in the cochlear nucleus (second-order neurons) are also able to respond to a particular phase of such high frequency tones. This response characteristic is termed phase locking. Because a single neuron can at maximum fire at about 1 kHz, the owl could either a) average over time, or b) sample the response of several neurons in order to use phase cues in high frequency signals. To discriminate between these possibilities, we determine the minimum duration of a stimulus that allows the owl to localize a sound source in azimuth. We test the owl's performance both behaviorally and electrophysiologically. We expect the convergence of multiple channels not to take place before the conversion of a time code (phase locking to monaural signals) to a place code (no phase locking, but tuning to time differences) in the third-order neurons of nucleus laminaris. The convergence could also arise at higher order nuclei such as nucleus ventralis lmnisci anterior (VLMas) or the auditory midbrain nucleus, mesencephalicus lateralis, pars dorsalis (MLD), where space-specific neurons form a map of auditory space.

As a first step in this investigation, we recorded from space-specific neurons in MLD, because these neurons are very sensitive to interaural time differences. The first recordings indicate that the owl can make use of a stimulus duration as short as 500 microseconds (may be even shorter), which corresponds to about 2-3 tonal cycles at 5-9 kHz.

260. Auditory Representation of Autogenous Song in the Song System of White-Crowned Sparrows

Daniel Margoliash

HVC (Hyperstriatum Ventrale, pars caudale) is a forebrain nucleus in the motor pathway for the control of song. Neurons in HVC also exhibit auditory responses. A subset of these auditory neurons in the white-crowned sparrow (Zonotrichia leucophrys) have been demonstrated to be highly selective for the individual bird's own (autogenous) song. By using multiunit recording techniques to sample from a large population, I found that the entire population of auditory neurons in HVC is selective for autogenous song. The selectivity of these neurons must reflect the song learning process, for the acoustic parameters of a sparrow's song are acquired by learning. By testing with laboratory-reared birds, I showed that HVC auditory neurons prefer autogenous song to the tutor model to which the birds were exposed early in life. Thus, these neurons must be specified at or after the time of song crystallization. Because song is learned by reference to auditory feedback, HVC auditory neurons may guide the development of the motor program for song. The maintenance of a precise auditory representation of autogenous song into adulthood can contribute to the ability to distinguish the fine differences among conspecific songs.

261. The Origin of Brain Sexual Dimorphism by Neuronal Growth, Atrophy and Death in the Zebra Finch

Eugene Akutagawa, Mark Konishi

The song control nuclei of the zebra finch brain contain more neurons of larger size in the male than in the female. This sexual dimorphism was thought to result from differential growth of neurons in the two sexes. We have studied the development of several forebrain nuclei involved in the control of song and find that the dimorphism arises from neuronal atrophy and death in the female brain as well as from an increase in cell body-size in the male.
Summary: Our research this past year is part of a long-term project aimed at understanding the mode of action of the ascending brain catecholamine systems of the rat in regulating brain reward mechanisms and motor activity. Considerable anatomical, physiological, and pharmacological evidence shows that these systems have the capacity to influence behavior maintained by positive reinforcement and the level of spontaneous motor activity of the animal. But which of the several catecholamine systems present in the CNS, and which of the several subdivisions in each system is involved in these particular functions is at present not known. Of particular interest are the dopamine (DA) systems originating in the ventral midbrain whose projections terminate in frontal cortex, striatum, nucleus accumbens, septum, and hippocampus. One of these, the nigrostriatal system, is implicated in the regulation of motor activity. Another, the mesolimbic system, is implicated in the regulation of emotionality.

We have studied the basis of reinforcing mechanisms in the CNS with the self-stimulation methodology. We use rats chronically implanted with electrodes aimed at the DA systems (regions of origin or terminal fields) and test for the motivational properties of brief electrical shock. Subjects are trained to self-apply the shock by pressing a lever placed on one of the walls of the test chamber. The depression of the lever results in the closing of a microswitch, which in turn causes the application of a brief electrical shock via the implanted electrode. This approach has revealed a wide distribution of brain reward sites. Other studies have revealed that this electrical reward can function in the manner of natural rewards such as food to promote learning. The widespread distribution of reward sites in the mammalian brain has raised the question of whether there is one or several reward mechanisms and what is the anatomical linkage that unites these disparate sites.

Our interest in motor regulation derives from two observations. One is that brain rewarding stimulation is accompanied by motor activation, but not at all reward sites, suggesting that the two systems involved are independent though interdigitated. The other observation is that specific damage of the DA systems leads to hyperactivity. We aim to determine what subpopulation of DA or CA neurons must be damaged neonatally to induce developmental hyperactivity. Damage which is inflicted neonatally leads to hyperactivity. We aim to determine what subpopulation of DA or CA neurons must be damaged neonatally to induce developmental hyperactivity and what is the physiological basis of this syndrome.

Our experiments are based on the use of two approaches. One involves the use of neurotoxins specific to the catecholamines to inflict damage to the CA systems. We have used these neurotoxins in combination with cytotoxins to gain insight into interactions between DA neurons and non-DA neurons present at rewarding sites. The other approach

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involves the use of electrophysiological methods to record extracellular neural activity following pharmacological activation or inhibition of DA activity. The objective is to establish relationships between the activity of subpopulations of DA neurons and the behavioral effects of pharmacological manipulations. In this way insight may be gained into the ways in which dopaminergic activity is transmitted, integrated, or modified within the local networks of each of the DA systems as a result of changes in CA-linked behaviors.

262. Anatomical Basis for Brain Reward in Substantia Nigra and Nucleus Accumbens of Rats with Brain Levels of Dopamine Depleted

T. Takeichi¹, Mamoru Umemoto¹, Marianne E. Olds

Dopamine (DA) neurons giving rise to pathways that innervate the forebrain have been implicated in the rewarding effects of brain stimulation. Mapping studies designed to delineate a reward pathway have shown a high correlation between sites rich in dopaminergic innervation and sites where electrical stimulation is rewarding. Pharmacological studies using DA-related drugs have shown that DA agonists facilitate responding to obtain the brain reward while DA antagonists suppress responding. Since these antagonists are known to interfere with the initiation of movements, questions have been raised about the basis of the effect of the DA antagonists on reward, specifically, whether the suppression of responding for the brain stimulus is due to interference with performance.

We have investigated the role of the ascending DA systems in brain stimulation reward with the lesion technique. In the first experiment, the effects of the neonatal depletion of DA levels in rat brain by treatment with the neurotoxin 6-hydroxydopamine (6-OHDA) on responding for the brain reward in substantia nigra, zona compacta, substantia nigra, zona reticulata, caudate nucleus and nucleus accumbens, were investigated. These areas give rise to or receive dopaminergic projections. In the second experiment, the cytotoxic substance kainic acid (KA) was injected in tissue below the electrode to destroy cell bodies but not fibers or terminals present at the reward site. This substance is a rigid analogue of glutamate, a putative neurotransmitter with receptors found on cell bodies, but not terminals. KA binds to the glutamate receptor, causes hyperexcitation of the neuron followed by swelling and eventual neuronal death. Our objective was to compare effects of 6-OHDA on reward via destruction of the dopaminergic innervation of the forebrain with those produced by the destruction of cell bodies only via KA injected at the site of stimulation.

Our results show that in rats with 92% of DA content neonatally depleted in cortex, 96% in striatum, and 28% in substantia nigra, reward mechanisms operate almost normally.

In contrast, the local injection of KA in tissue below the electrode tip in controls or DA-depleted subjects permanently suppressed responding for the electrical stimulus. KA injected contralaterally to the reward site did not interfere with responding for the brain stimulus on the ipsilateral side. Apparently, the integrity of DA target neurons in nucleus accumbens and substantia nigra is necessary to induce and support brain reward in these regions.

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263. Comparison of the Hyperactivity Induced in the Adult Rat by Neonatal Treatment to Deplete Brain Catecholamines

Marianne E. Olds, Arthur Yuweiler¹

Spontaneous motor activity in the rat increases for the first two weeks of life and then declines during the third and fourth week to adult levels. The high level of activity during the first two weeks of life has been associated with the maturation of the central catecholamine systems while the decline during the third and fourth weeks with the maturation of the cholinergic and gabaergic systems. Neonatal treatment with the neurotoxin 6-hydroxydopamine (6-OHDA) induces hyperactivity which may be due to an extension of the catecholamine development period or to a delay in the maturation of the cholinergic or gabaergic systems. In many studies of neonatal hyperactivity, efforts to understand the neurochemical basis of the motor dysfunction have focused almost exclusively on the changes of catecholamine levels in the neonate and the young adults, not on the changes during maturity. In our own previous work (Fobes and Olds, 1981), we have reported that high doses of 6-
OHDA injected bilaterally and neonatally into the lateral ventricles lead to the development of explosive running behavior during the third and fourth weeks of life. We also reported that the hyperactivity extends into maturity and old age. This persistence prompted a longitudinal study of the hyperactivity aimed at determining the respective roles of DA and NE.

On day 5 after birth, male and female rat pups were pretreated with pargyline or desmethylimipramine (DMI) (pargyline is a monoamine oxidase inhibitor given to potentiate the action of 6-OHDA which was injected 30 min later; DMI is a compound preferentially inhibiting uptake into noradrenergic neurons and as such protects these neurons from the toxic action of 6-OHDA). Each subject received two injections, one in each lateral ventricle. Controls were pretreated with pargyline or desmethylimipramine, and then were given intraventricular injections of saline.

Spontaneous activity was measured in a stabilimeter at ages 30-31, 42-45, 60-63, 75-77, and 120-122 days. In addition, activity in a group of controls and a group of pargyline + 6-OHDA subjects was measured at 254 days of age.

Assays of catecholamines in different brain regions were determined when the animals were 150 days old. The results show that in the pargyline + 6-OHDA group the levels of NE were reduced in frontal cortex (7% of control levels), caudate nucleus (21%), and hippocampus (14%). The NE levels were unchanged in hypothalamus, ventral midbrain and pons. The DA levels in the pargyline + 6-OHDA animals were depleted in caudate (8% of control levels), ventral midbrain (32%) and they were unchanged in hypothalamus and pons.

In the DMI + 6-OHDA group, the NE levels were reduced in caudate (25% of controls) and elevated in hippocampus (188%). The DA levels were depleted in caudate (3%) and ventral midbrain (22%).

The neonatal treatments with pargyline + 6-OHDA and with DMI + 6-OHDA resulted in long-term hyperactivity, but the syndrome was of larger magnitude and more permanent in the pargyline + 6-OHDA group. The evidence implies a facilitatory role for NE but a critical role for DA in developmental hyperactivity in the rat.

References:

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264. Electrophysiological Study of Nigral Non-dopamine Neurons during Dopamine Activation

Marianne E. Olds

The substantia nigra (SN) is a structure lying in the ventral midbrain with two subdivisions that contain different types of transmitter-identified cells. In the dorsal subdivision, referred to as the zona compacta (SNC), are the cell bodies that give rise to the nigrostriatal system. These cell bodies, their axons, and their terminals contain the neurotransmitter dopamine (DA). These neurons innervate the caudate-putamen. Experimental and clinical data implicate this pathway in the regulation of motor activity. In the ventral subdivision, referred to as zona reticulata (SNR), are cell bodies of neurons also implicated in motor regulation. These cells do not contain DA, but some of them contain DA receptors on the cell membrane. They receive a massive projection from the striatum, and they send projections to various pre-motor and motor nuclei. An area adjacent to the SN, the ventral tegmental area (VTA), also contains DA and non-DA neurons, but the two types are not segregated in different subdivisions. Recent data suggest that the nigrostriatal DA pathway mediates information relevant to head movement and posture while the VTA DA pathway to nucleus accumbens carries information about locomotion. The DA neurons of the SN, and probably also of the VTA, have long dendrites which release DA in the SN-VTA. The dendritic-released DA is thought to regulate the activity of neighboring DA-containing neurons (by a local feedback mechanism) and other, non-DA neurons located nearby. Little is known, however, of the effects of DA activation on these non-DA neurons in the unanesthetized, behaving animal. To gain insight into the interactions between DA and non-DA neurons, we have recorded cellular activity in the SN and VTA of the rat given d-amphetamine to induce release of DA, or apomorphine to induce direct activation of the DA receptor.

The results show an increase in firing of non-DA neurons of the SN-VTA. Not all non-DA neurons,
however, are excited. Some show no effects, suggesting functionally different subpopulations. The excitation is blocked or attenuated by pre-treatment with haloperidol, a DA receptor blocker. Experiments carried out in a rat lightly anesthetized rat (urethane) so that it can move its head but not engage in locomotion show a reduction in the number of amphetamine induced excited non-DA neurons.

Apomorphine, a direct stimulant of the DA receptor, also caused excitation of non-DA neurons in SN and VTA. These findings, viewed in the context of the available evidence showing that DA neurons are inhibited by d-amphetamine and apomorphine, provide direct evidence of opposite effects of released DA on two functionally related populations of neurons in the ventral mesencephalon.

PUBLICATIONS


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Summary: Our research continues to center on hemispheric specialization and left-right cross integration in human subjects and monkeys who have undergone either surgical disconnection of the cerebral hemispheres or hemispherectomy. In humans, we have emphasized nonverbal cognitive processing and its subcortical transfer, along with the mechanisms underlying auditory dichotic testing techniques and the influence of task difficulty on hemispheric interrelations. In monkeys, evidence for complementary hemispheric specializations has been greatly strengthened. Related theoretical work is focused on the scientific and humanistic implications of the macro-determinist paradigm upheld in cognitive science.

265. Dichotic Experiments Suggest Secondary Center Within Each Cerebral Hemisphere

Polly Henninger Pechstedt

Last year's finding of a shift in ear advantage with increasing task difficulty in a right hemispherectomy subject (Biology 1984, Abstract No. 227) was contradictory to traditional beliefs that left ear report reflects involvement of the right hemisphere, that ipsilateral pathways are suppressed in dichotic testing, and that suppression is a function of stimulus characteristics. Furthermore, preliminary investigation using musical stimuli suggested that this subject had greater difficulty labeling songs that were presented to his left ear than to his right, a surprising result given the absence of his right hemisphere. These findings, the well-known unity of behavior of the split-brain subjects, and the evidence that both halves of the body and both sides of the perceived world are represented within each hemisphere by ipsilateral and contralateral projections led Sperry to suggest that there are two processing centers within each hemisphere: a primary center for contralateral representations and a subsidiary or secondary center for the ipsilateral functions.

To investigate this hypothesis, the subject was further tested with the digits tape biased to the left ear and asked to report left or right ear digits only. In both cases he reported left ear digits at the outset and shifted to right ear digits. The timing of the shift varied with the instruction. In the latter test, as he shifted he reported that the sounds shifted from his left to his right ear. Apparently, the higher volume in the left ear gave dominance to the ipsilateral path-
ways and a secondary center, but as the cognitive load increased, the primary center took over and the subject shifted to the contralateral input. In monaural testing of the left ear, he initially guessed and then suddenly shifted to the correct answers for the remainder of the test. These results suggest two distinct processing centers within the single hemisphere, that inputs from each side of space project to different centers, and that the ipsilateral, secondary center is specialized for functions normally the domain of the opposite hemisphere. Further study is planned to distinguish between secondary center functions and those that can only be performed by the more powerful contralateral hemisphere.

266. Task Difficulty Induces Increasing Right Ear Advantage in Normal Subjects

Polly Henninger Pechstedt, Tad White

Last year we reported dichotic test results in which a shift in ear advantage with increasing task difficulty was observed in a small group of normal adults, and in hemispherectomy and commissurotomy subjects (Biology 1984, Abstract Nos. 226 and 227). The task was to identify by writing with the right hand one to four digits presented simultaneously to each ear with the volume raised by 5-10 dB in the left ear to offset the typical right ear advantage. This year we tested our finding by presenting the task to a larger group of normal subjects and by incorporating cognitively more demanding tasks to see if the shift could be induced by increasing the load on the left hemisphere.

Three groups of normal adult subjects were given the digits test. Subjects in the first group (n = 24) were instructed to simply write the digits as before, in the second group (n = 9) to write the digits in sequence from lowest to highest, and in the third group (n = 8) to add the digits mentally, write the sum and then write the digits they recalled.

In contrast to last year, subjects instructed to simply identify the digits did not show a change in ear advantage. Subjects given the cognitively more demanding tasks (to write the digits in sequence and to sum the digits first), however, showed a significant increase in relative right ear report as the number of digits increased (t-tests, p <0.05).

The significant increase in the right ear advantage indicates that it is possible in normal subjects, as in the clinical subjects, to alter the competition between the ipsilateral and contralateral pathways from the ear to the dominant hemisphere by increasing the cognitive demands of the task. These findings suggest that the mechanism that causes ipsilateral suppression is present, and similar, in both populations. It does not depend on the presence of the corpus callosum; it can occur within a single hemisphere and it is influenced by the cognitive demands on that hemisphere.

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267. Top Performers Process Input to the Nondominant Hemispheres

Polly Henninger Pechstedt

To explore the relationship between musical and verbal processing, 24 right-handed subjects, half with musical training and half untrained, were given dichotic digit and melody recognition tests. Both tasks evoked the typical ear advantage (a right ear advantage on the verbal test for both groups; a left ear advantage for untrained subjects on the music with a trend for training groups to show opposite ear advantages). The degree of correlation between performance and the laterality index on each test was calculated. For both tests there was a significant correlation between performance and the relative advantage of the ear contralateral to the hemisphere nondominant for that task: higher performance on music was associated with a right ear advantage; higher performance on verbal material was associated with a left ear advantage. Trained musicians tended to have smaller relative ear differences on both tests.

Subjects were grouped according to their ear advantage on each test. Scores for each test were normalized and subjects were compared on their combined performance on both tests. Subjects with the normal pattern of laterality (a right ear advantage for digits; a left ear advantage for melodies) performed the worst. Musically trained subjects with no ear difference on one test and an ear advantage contralateral to the nondominant hemisphere on the other performed the best. They identified the input to the hemisphere that is nondominant for the task equally well or better than the input to the dominant hemisphere. These findings suggest that higher performance results from using the capacity of both hemispheres and that this requires inhibiting the
dominant hemisphere and attending to the input to the nondominant. It appears that the ability to use the input to the nondominant hemisphere may result from responding to the memory demands as opposed to the stimulus demands of a task and that musical training improves the ability to use the nondominant hemisphere for processing aural material.

Lastly, because traditional measures of dominance confound laterality and performance, these results must be considered tentative. Further testing using more difficult tasks and matching subjects for performance is necessary.

268. Does Caltech Cause Burnout?

Polly Henninger Pechstedt

Sparked by recent graduate Marc Buffet's hypothesis that Caltech causes burnout, this study was undertaken to test whether the Caltech educational experience decreases activity in the dominant cerebral hemisphere. The students in a section of Bi23 were grouped by their years at Caltech, older (juniors and above, n = 7) and newer (freshmen and sophomores, n = 5) and compared on ten dichotic tests (seven verbal and three musical). Across all tests the newer group outperformed the older group (sign test, p <0.01). These differences were attributable to lowered performance levels in the dominant (left for verbal, right for musical) hemisphere: older subjects were significantly worse than newer subjects in the dominant hemisphere on six tests (t-test, one-tailed, p <0.1), and showed similar but not significant results on the final test, while the two groups did not differ significantly in the nondominant hemisphere on any of the ten tests. These results suggest that a Caltech education is decreasing mental capacity by fatiguing the hemisphere most involved in a task.

An analysis of the individual tests supported this conclusion. When the volume was raised in the right ear and the subjects were asked to simply report the digits, older subjects made as many errors in the right ear as the left (newer made one third as many, p <0.05). Even zapping the left hemisphere by raising the volume in the contralateral ear did not help the older students. The greatest difference between the groups was on the test designed to demand the most left hemisphere resources, a verbal test in which the volume was raised in the right ear and subjects were required to add the digits mentally before writing what they recalled. Older subjects made twice as many errors as newer subjects and three times as many errors in the dominant hemisphere (p <0.001). The results of both tests suggest that the lowered performance is not due to attentional or motivational factors but to left hemisphere burnout.

If replicated, this study suggests that decreased hemispheric capacity is a consequence of a Caltech education.

269. Right Hemispheric Superiority in Monkeys for Discriminating Faces

Betty A. Vermeire, Charles R. Hamilton

Sixteen split-brain monkeys have completed three experiments that examine the ability of the left and right hemisphere to learn, remember, and generalize to novel exemplars, eight discriminations based on the identity or expression portrayed by photographs of the faces of other monkeys (Biology 1984, Abstract No. 229). The experimental designs were balanced for sex, handedness, side of surgical approach, and problem type. Although the right hemisphere learned more readily, it was not significantly superior to the left in this experiment. However, the right hemisphere was significantly better at remembering the eight facial discriminations when tested about five months later (p <0.02). Furthermore, the right side also was more proficient at correctly recognizing new photographs of the individuals or expressions that had been learned (p <0.02).

Eight additional monkeys are now being tested in the same way. Although the results are not yet complete, the data confirm and extend the previous findings. Taken together, learning by all 24 monkeys was significantly better by the right hemisphere (p <0.05). Furthermore, for the 20 monkeys tested so far, memory is still significantly stronger in the right hemisphere (p <0.02). Because the mnemonic tests consistently gave larger and more reliable hemispheric differences than did the learning tests, they may provide a particularly useful measure of cerebral asymmetries in subsequent experiments.

The right hemisphere of human subjects is also superior to the left for many aspects of facial recognition, suggesting the lateralized mechanisms in
monkeys may be homologous with those in human beings. Interestingly, female monkeys showed larger and more significant hemispheric differences than male monkeys in these and other experiments. Perhaps this reflects a greater need for subtle communication of social and affective information by female monkeys in simian society.

270. Complementary Hemispheric Superiorities in Monkeys

Charles R. Hamilton, Betty A. Vermeire

The ability of monkeys to discriminate stimuli differing in spatial position or orientation is better represented in the left hemisphere of monkeys than in the right (Biology 1984, Abstract No. 228). The most reliable test of this visuospatial ability we have found requires discrimination between lines tilted by 15° or less in the frontal plane (Biology 1982, Abstract No. 206). For 19 monkeys tested so far, the left hemisphere learned four problems of this type significantly more readily than did the right (p < 0.01).

Twelve monkeys have learned both these visuospatial discriminations and the facial discriminations described in the preceding abstract. This allows a direct comparison of hemispheric differences within each subject and effectively controls for artifactual asymmetries induced by the surgery or testing procedures. Eleven monkeys showed relatively greater left hemispheric superiority for the spatial task than for the facial one (p < 0.001). In eight of these monkeys, the absolute side of lateralization was opposite for the two types of discriminations and in the expected directions. Two of the remaining subjects showed no lateralization for one of the two types of test and showed reversed laterality for one type. Overall, these results indicate that monkeys, like humans, possess complementary specialization of their two cerebral hemispheres for performing different behavioral functions. Our ongoing experiments are attempting to determine how similar these examples of lateralization in monkeys are to those found with human subjects. It appears that this system will provide a useful model of human-like cerebral lateralization that may be used for investigating the biological basis of hemispheric specialization with methods not suited to experiments with human subjects.

271. Stereopsis Near the Vertical Meridian of the Visual Field

Charles R. Hamilton, Katrin M. Rodriguez, Betty A. Vermeire

Stereopsis along the vertical midline of the visual field in higher mammals is often thought to require interhemispheric connections because the receptive fields of disparity-sensitive neurons near the vertical meridian of the cortical representations in cats and monkeys appear to utilize commissural connections. However, very few behavioral tests of midline stereopsis in humans or animals have been made to verify this possibility.

We have tested five patients with surgical division of the corpus callosum and the anterior and hippocampal commissures for their ability to detect stimuli displayed as dynamic random dot stereograms. The stimuli were 2° across and had a crossed disparity of 10, 20, or 40 minutes of arc, which produces a perception of the target in front of the background, or similar stimuli with uncrossed disparities, which produce a perception of the target behind the background. The target was flashed for about 200 milliseconds at unpredictable positions along the horizontal meridian between ±10° of fixation and along parallel meridians 5° above or below fixation. The subject's task was to point to the position of the stimulus with the left hand for targets on the left of center and conversely for the right. The percentage of correct responses at each position was determined and used as an estimate of stereopsis.

Two patients, NG and RY, showed striking, reproducible stereoscopic defects within ±2° of the vertical meridian of the tests with crossed disparities, in keeping with the presumed commissural contribution to midline stereopsis (Biology 1984, Abstract No. 231). However, the other three patients, LB, AA, and NW, showed little evidence of midline deficits. Subsequent tests with uncrossed disparities failed to reveal midline losses for NG (RY has yet to be tested). Thus, the cerebral commissures do not seem necessary for midline stereopsis tested with dynamic random-dot stimuli.

Eight normal subjects have been tested in the same way. Surprisingly, three of them showed midline deficits in stereopsis similar to NG and RY. Therefore, there is no compelling need to relate the
patients' deficits to their surgery. However, the midline deficits in all subjects, split-brain or normal, may still be attributable to decreased commissural function. It is plausible that the extent of commissural participation in stereopsis is highly variable across subjects, perhaps reflecting the allocation of commissural connections to specific functions during development. Further tests of stereopsis of these subjects with other forms of stereopsis and non-stereoscopic stimuli are needed to clarify these and other issues.

\[1\] Undergraduate, California Institute of Technology.

272. Simple Reactions to Lateralized Flashes of Light by Commissurotomized Patients

Justine Sergent, Jay J. Myers

Simple reactions to lateralized flashes of light provide the opportunity to examine interhemispheric transfer of information by comparing reaction times (RT) obtained in conditions where input reception and response production take place within the same hemisphere (ipsilateral response) to RTs obtained in conditions where input reception and response production take place in different hemispheres (contralateral response). Two complete forebrain commissurotomized patients (NG and LB) had to produce a finger, blowing, or verbal response to a light appearing in either the right or the left visual field. A normal subject was tested in exactly the same experimental conditions. The three subjects displayed qualitatively similar patterns of results overall. The finger task yielded a highly significant interaction between visual field and responding hand, and the RT difference between ipsilateral and contralateral responses was of the order of 30 and 50 ms for each patient, respectively, suggesting that subcortical transfer of information is highly inefficient in the manual task compared to a callosal route yielding transfer duration of 2-3 ms. By contrast, the blowing and the verbal tasks resulted in no visual-field difference, even though the latter requires the specialized mechanisms of the left hemisphere for speech production. The results suggest that different pathways can subserve subcortical transmission of information across the hemispheres, and that a verbal response may be initiated in subcortical structures to which both hemispheres have equally efficient access before being organized within the left hemisphere.

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273. Similarity of Separated Hemispheres for Cognitive Association of Visual Material

Alice M. Cronin-Golomb

A recurring question in split-brain research concerns the preference versus ability of the hemispheres to process various types of stimulus material. As small, forced-choice formats may artificially limit or amplify a hemisphere's ability or propensity to perform a cognitive task, the following match-to-sample experiment was designed to include a large response array of an unrestricted number of "correct" answers. Of 20 Peabody picture cards comprising the array, ten matched the sample on the basis of appearance, category, function, or an abstract relationship, and ten were unrelated to the sample.

The randomly-ordered cards of the response array were placed, one at a time, either within the same visual hemifield (VF) as the sample for subjects NG, LB and AA (intrahemispheric test) or in the opposite VF (interhemispheric test), using the lateral limits technique (Myers and Sperry, 1982). The subject was instructed to point to all the cards that were related in any way to the sample. The chosen subset was presented again, by pairs, until all were ranked according to the nearness of relationship with the sample. One trial (20 cards) was presented in free vision for instructional purposes, and three trials each to the left VF (LVF) and right VF (RVF).

RVF trials elicited a larger number of responses/trial ($X = 4.3$) than LVF trials ($X = 2.9$), both of which were lower than free vision responses ($X = 8.7$). The two hemispheres were similar in the types of associations made, with NG and LB matching for abstract as well as concrete relationships, and AA's responses being few in number and highly concrete. The hemispheres chose many of the same cards ($X$ common responses $= 2.9$, of $X$ total responses/hemisphere $= 3.1$), though rank orders were not consistent.

On the interhemispheric test, the number of responses was very similar in the left to right hemisphere and right to left direction ($X = 4.5$) with a $X$ of 3.1 responses in common for the two directions. All
types of responses were represented, and rankings were rather consistent.

In summary, when split-brain subjects are allowed to choose from a large array an unrestricted number of pictorial matches to a sample, both hemispheres make matches based on appearance, function, category membership, and abstract relationship, though the left hemisphere chooses more responses than the right, and neither chooses as many as do the two together in free vision. Subcortical channels may also support the many types of association upon which matches may be based, as revealed under interhemispheric test conditions.

Reference:

274. Perception of Ambiguous Apparent Motion Displays by Commissurotomy Patients

Vilayanur S. Ramachandran, Alice Cronin-Golomb

If two spots of light are flashed simultaneously on diagonally opposite corners of a square and followed by two spots presented in the remaining two corners, one can either see vertical or horizontal motion and the display is ambiguous. If several ambiguous figures are viewed simultaneously, they tend to become uniformly oriented (either horizontal or vertical), suggesting that a "global" strategy is used to resolve ambiguity, even if the displays lie on different sides of the vertical meridian.

After commissurotomy, certain kinds of visual cross integration are preserved (Trevarthen and Sperry 1973; Myers and Sperry, 1985). We have now studied motion perception in these subjects.

First, we presented either a light spot that jumped 14° across the vertical meridian left to right (L-R) or right to left (R-L) or two spots that appeared simultaneously. Three subjects could accurately discriminate L-R from R-L and either of these from simultaneous presentation (90% of 80 trials). Next, two subjects (AA and LB) fixated the center of a single ambiguous figure (described above) subtending 4°. Surprisingly, they could see either of the two percepts horizontal or vertical oscillation of the spots. When seeing horizontal oscillation, they must be inhibiting or vetoing the vertical motion signal. Hence, the circuitry responsible for this bistability (and inhibition) may not involve the forebrain commissures.

Finally, we presented several such ambiguous figures simultaneously on the screen on both sides of the fixation point. In normal individuals, such figures become consistently oriented, but in LB and AA the direction of perceived oscillation was often different in the two hemifields (as indicated by the two hands). They could "will" a perceptual switch in one hemifield without involving the other hemifield. Hence, global coupling of ambiguous motion displays may require intact forebrain commissures but the perception of simple apparent motion sequences is probably mediated by the "second" visual system.

References:

PUBLICATIONS


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Summary: Our group is interested in how visual information is processed in the cerebral cortex. The primary focus of our research is the macaque monkey, whose sense of vision is comparable to our own and whose cerebral cortex contains a mosaic of many distinct visual areas in the occipital, temporal, and parietal lobes. We use a combination of physiological and anatomical techniques to identify and chart the extent of different areas, to trace the flow of information between areas, and to ascertain the functional properties of their constituent neurons.

This past year has been marked by progress on many fronts. We have begun a collaborative venture with Bela Julesz, Visiting Professor from AT & T Bell Labs, to study the physiological basis of texture vision in monkeys. His psychophysical studies on the perception of texture patterns have demonstrated that the human visual system can operate in two distinct modes. One mode involves "pre-attentive," massively parallel screening of the visual world to look for objects or items of potential interest; the other mode uses attentive scrutiny to analyze such objects in finer detail. Our physiological experiments on monkeys are aimed at finding neural correlates of these two modes of vision; the preliminary results obtained to date look highly promising.

In recent years several surprising features concerning the basic organization of visual areas have emerged from work done in our own and other laboratories. One feature is that some visual areas lack a complete representation of the visual cortex, yet are well-defined entities by a number of independent criteria. We have previously found this to be the case for a pair of areas, V3 and VP, one of which is concerned only with the lower part of the visual field and the other with the upper half. During the past year we have extended this approach to a different region and have found evidence that another pair of areas, V4 and VA, may show a comparable asymmetry in its organization.

Another organizational feature is that individual visual areas may be broken up into an orderly mosaic of numerous small subregions that can be visualized using anatomical techniques such as histochemical staining for the enzyme cytochrome oxidase. We have examined the functional significance of this arrangement by a combined approach of physiological recording and pathway tracing for the different subregions of visual area V2. Our results demonstrate that these subregions differ in the targets to which they project and in the types of visual information they transmit. This provides clear evidence for the hypothesis of distinct "functional streams" for the processing of information about motion, form, color, and depth. In our effort to explore this hypothesis in greater detail, we are incorporating a new experimental method that uses optical rather than electrical measurements to assay neural activity. This approach, involving application to the cortex of dyes whose fluorescence changes as a function of membrane potential, offers a promising way of monitoring activity of neural ensembles with high spatial and temporal resolution.
We have also made progress in several phases of our long-term goal of bringing computer technology to bear on neuroanatomical problems. One important accomplishment is the first computer-generated two-dimensional map of the cerebral cortex. Heretofore, we have relied on a tedious manual procedure for constructing these maps, but the availability of a computerized procedure will substantially improve their accuracy and quality. Another major step has been the establishment of a computerized anatomy facility for use by neuroanatomists within the Division of Biology. This facility includes a microscope with a motorized stage, and a direct link to a computer for storage of relevant anatomical data. In the coming year we plan to develop software that will permit the generation of three-dimensional reconstructions from a series of sections as well as translation of data onto the aforementioned two-dimensional cortical maps.

An independent facet of our research program concerns the plasticity of synaptic connections at the mammalian neuromuscular junction. We use the soleus muscle of the rabbit as a model system for understanding the phenomenon of synapse elimination, whereby synaptic contacts are lost in large numbers during the normal course of postnatal maturation. One major topic of interest is the relationship of synapse elimination to the occurrence of separate fast- and slow-contracting fiber types interspersed within each muscle. Interestingly, we have found that multiple inputs are retained significantly longer on slow muscle fibers than on fast fibers. We are also interested in the role of electrical activity in influencing the fate of synapses competing for survival. Our results to date suggest that synapses rendered inactive by application of tetrodotoxin are essentially as effective as normally active synapses in this competition. This result stands in surprising contrast to a number of studies suggesting a large role for neural activity during development.

275. Anatomical and Physiological Heterogeneity of the Second Visual Area, V2, of the Macaque

Edgar A. DeYoe, David C. Van Essen

The cerebral cortex of the macaque monkey contains over a dozen distinct areas that are involved in vision. The exact function of each area has yet to be determined, but one recent advance has been the realization that individual areas may themselves be functionally heterogeneous. By staining cortical tissue for the mitochondrial enzyme, cytochrome oxidase (CO), it is possible to observe a "polka dot" pattern of darkly stained spots within tangentially cut sections of the first visual area, V1. Within the second visual area, V2, the same procedure yields a pattern of dark stripes that are alternately thick and thin and are separated by pale interstripe regions. It is known that neurons within the CO polka dots (blobs) of V1 have physiological properties that differ from those of cells outside the blobs. In addition, the projections from blob and non-blob cells are selectively distributed to the different CO stripe compartments in V2, thus suggesting that they too may be physiologically heterogeneous.

We have investigated the anatomy and physiology of the V2 stripe regions by combining single neuron recordings with CO histochemistry and pathway tracing techniques. In this manner we are able to simultaneously assess the physiological properties and efferent connections of cells in the different CO stripe regions.

We have found that the connections from V2 to two of its major targets, areas V4 and MT, arise from separate groups of cells. Those projecting to MT reside mainly within the thick CO stripes whereas those projecting to V4 are found mainly in the thin and interstripe regions. Furthermore, neurons in these different subregions tend to respond selectively to different aspects of a simple visual display. Cells within those regions projecting to MT are preferentially involved in the processing of information about motion, stereoscopic depth and the orientation of contours. On the other hand, neurons in those CO regions projecting to V4 are often concerned with color but not motion. Like the MT-projecting cells, however, they too process some information about stereoscopic depth and contour orientation.

Our data, in combination with that from other laboratories, suggest that the second visual area may be composed of multiple, physiologically distinct subregions, each of which may have its own unique role to play in the creation of our visual perceptions.
276. Two Topographically Organized Visual Areas in Ventral Extrastriate Cortex of the Macaque Monkey

Daniel J. Feildman, Edgar A. DeYoe, David C. Van Essen

Over the past several years we have used a multifaceted neuroanatomical and electrophysiological approach to show that the strip of cortex immediately anterior to V2 consists of two separate visual areas, V3 and VP, rather than the one visual area previously recognized (Biology 1981, Abstract Nos. 233, 234). An important corollary of this finding is that V3 only represents the lower visual field while VP only represents the upper visual field. The differences in physiology between these two areas indicate that visual information is processed differently in the upper and lower visual fields.

We have now addressed the same basic issue as it relates to a large region anterior to V3 and VP. This strip consists of one region, V4, known to receive inputs from dorsal V2, and a more ventral (but still contiguous) region known to receive inputs from ventral V2. To ascertain whether the ventral region should be considered as part of V4, we have used electrophysiological mapping techniques and pathway tracing techniques to assess the topographic organization and connections of this region.

We mapped the representation of visual space in the cortex anterior to VP in ventral extrastriate cortex in 31 microelectrode penetrations in four hemispheres of three monkeys. The results indicate that there is not just one, but two separate topographically organized representations of the superior contralateral quadrant in cortex anterior to VP. The first region adjoins VP along the representation of the superior vertical meridian. Provisionally, we have named this region VA, the ventral anterior area, and suggest that it is probably distinct from V4. In front of VA is a second region that shares a representation of the horizontal meridian within VA and contains a representation of the vertical meridian at or near the posterior border of PIT, the posterior inferotemporal area. Provisionally, we have named this new area PT, the posterior temporal visual area. Each of these two regions represents central fields laterally and peripheral field medially in cortex.

To determine if VA and V4 can be distinguished on the basis of cortical connections, we used a double retrograde labeling technique to study the connections of VA and V4 in the same hemisphere. Both injections produced clear stripe-like patterns in V2. In addition to connections with several well-known visual areas, these injections produced interdigitating patterns of label in the intraparietal sulcus and across a large portion of the posterior inferotemporal area. Both injections produced widely separated foci of label in PIT, suggesting that it may contain several distinct subdivisions. In addition, labelled foci from these upper and lower field injections were almost completely non-overlapping. This suggests a crude topographic organization in the major afferents to PIT. This result contrasts with previous single unit recordings from PIT in which most receptive fields include both upper and lower fields.

277. Some Neurophysiological Correlates of Texture Perception

Edgar A. DeYoe, Dov Sagi, James Knierim, George J. Carman, David C. Van Essen

Why is it so hard to find the needle in a haystack? Why is the brush-bound partridge invisible until it moves? The problem is lack of contrast. The coloring of the partridge and the shape of the needle make them match the texture of their surroundings. If we substitute a golf ball for the needle or startle the bird into motion, a contrast between textures is created and the objects become visible. In fact, such differences can "grab" our attention, causing us to look directly at the object without having to laboriously search for it. How the brain accomplishes such a feat is unknown, but we are trying to find out how by studying the activity of single neurons in the cortex of monkeys while they view texture patterns on a television screen.

It is already known that many neurons in visual areas of the cortex respond selectively to the orientation or motion of an elongated bar of light on a blank background. One of the things we have done is to test single neuron responses when the bar (target) is surrounded by other bars (surround) of the same or different orientation, i.e., when it matches or contrasts with the surrounding texture. We have found some cells that, in fact, respond poorly when the target matches the surround but respond well when the target
and surround are contrasting. For other cells, however, the effect of the surround is more complex and depends on the specific orientations of target and surround.

We have also tested the responses of neurons to the motion and orientation of elongated texture borders. In this case, for example, the surround might consist of seven rows of similarly oriented line segments. A target consisting of one row of orthogonal line segments successively replaces each of the surround rows, producing the perception of a moving texture border. Though many cells respond to the orientation of the individual target elements, a few are selective for the orientation of the border regardless of the orientation of the individual elements. Such cells could underlie our ability to see the trees for the forest.

278. Optical Studies of Electrical Activity in Sensory Cortex

Harry S. Orbach, David C. Van Essen

Changes of absorption and fluorescence of certain membrane-bound dyes are linearly proportional to changes in membrane potential. Based on this, optical methods can be used to measure activity in the mammalian brain. The mean electrical activity in many adjacent areas can be monitored by projecting an image of the brain onto an array of about 100 photodetectors and measuring the photodetector outputs.

We are interested in applying this technique to mammalian sensory cortex with the aim of monitoring the activity of many sites simultaneously, measuring changes in electrical activity with submillisecond time resolution, having good spatial resolution, and having the ability to repeat the experiment on the same preparation with a variety of stimuli. In a sense, this would be the equivalent of real-time 2-deoxyglucose measurements.

This technique has already been used in experiments on tissue slices, salamander olfactory bulb, frog optic tectum, rat visual cortex and rat somatosensory ("whisker barrel") cortex. In the rat whisker barrel system, stimulating two different whiskers excited two localized regions of cortex whisker barrels which were observed as two distinct regions of optical activity.

We have set up such an optical system here and have tested it, measuring signals from salamander olfactory cortex and rat visual cortex. We will soon select a suitable preparation (offering large optical signals) which will probably be primary and/or secondary visual cortex of a small mammal. We will then proceed to address questions of functional organization and anatomy at a scale of about 100 µm. This is the scale at which a variety of stimulus parameters (e.g., orientation, direction, color) are mapped systematically across the surface of the cortex. The optical technique is uniquely qualified to examine the detailed arrangement of such local mappings by presenting the animal with varied stimuli and seeing the resultant patterns of cortical activity (analogous to determining the functional properties of single cells by monitoring spike activity in response to stimuli).

We also hope to use the technique in topographic mapping of cortical areas, in tracing connections within and between areas, and in examining dynamic features of cortical activity.

279. Computational Algorithms for the Production of Two-Dimensional Maps of Cerebral Cortex

George J. Carman, David C. Van Essen

In order to improve upon existing manual techniques for the construction of two-dimensional, unfolded representations of the cerebral cortex (Van Essen and Maunsell, 1980), we are developing a set of computational algorithms that will allow for the production of such maps by computer. Our procedures use regularly spaced histological sections to create a lattice of points that serves as a three-dimensional reference surface. For each point on this reference surface, a corresponding point is created on a two-dimensional map surface in a fashion that preserves topology. A metric of local distortion termed the energy is determined for each point from a comparison of the local geometry on the map with the corresponding geometry on the reference surface. The sum of energies of all points yields a metric of global distortion that is minimized in order to generate an optimal map of the reference surface.

To find this global energy minimum, we subject the map to simulated annealing (Kirkpatrick et al., 1983). In our implementation of this iterative algorithm, random displacements of points are attempted within the plane of the map. Displacements resulting in a more accurate local geometry (decrease in energy) are
always accepted, while those resulting in a less accurate local geometry (increase in energy) are accepted with a probability dependent upon a control parameter termed the temperature. Initially, the temperature is set high to allow positions of points on the map to become highly randomized. The temperature is then gradually reduced over successive iterations, allowing the points to approach their optimal local geometries and resulting in the emergence of an optimal global conformation of the map.

These procedures have successfully generated maps of mathematical surfaces, demonstrating that they can maintain topological order while generating an unfolded, two-dimensional map of a three-dimensional object with minimal distortion. We are extending these procedures to the mapping of large regions of visual cortex of the macaque monkey. Although many refinements remain to be incorporated, the results obtained thus far demonstrate that these algorithms provide a practical means for the computation of unfolded, two-dimensional maps of the cortex.

References:

280. Immunocytochemical Identification of Visual Areas in the Macaque Monkey

David C. Van Essen, Susan Hockfield

Visual areas in the cerebral cortex differ from one another in a variety of ways: each has a characteristic pattern of connections, both local and long distance; and many areas can also be distinguished by their characteristic architecture, as seen with classical myelin and/or Nissl stains as well as more modern histochemical procedures for enzymes and neurotransmitter-related substances. Presumably, these areas also must have characteristic markers that are used during development for recognition processes needed to establish specific connections. Immunological methods provide an obvious hope for identifying such markers.

We have begun to address this issue for primate extrastriate cortex using a monoclonal antibody, CAT 301, generated by Hockfield and MacKay at Cold Spring Harbor. The antibody was made against cat spinal cord, but immunoreactivity for it turns out to have a remarkably interesting distribution in the primate visual pathway. In primary visual cortex (V1) the antibody specifically labels layers 4B and 6 (Hendry et al., 1984). In extrastriate cortex of the macaque monkey, we have found a distinctive trilaminar pattern of labeling in area MT, which receives inputs from layers 4B and 6 of V1. There is also a bilaminar labeling pattern in cortex immediately medial to MT, in the region of area MST (the medial superior temporal area), which we have previously identified as a major target of MT. Further anatomical experiments will be necessary to establish whether CAT-301 labeling is precisely co-extensive with MST. Finally, we have found a patchy distribution of CAT-301 reactivity in area V2; we are currently investigating whether this patchiness is correlated with the characteristic stripe-like sub-regions distinguishable within V2 on the basis of cytochrome oxidase histochemistry (see Abstract No. 275).

These findings indicate that immunocytochemical methods can provide distinctive markers for functionally important subdivisions of cerebral cortex. The number of such markers and the degree to which individual markers are distributed widely (but nonetheless specifically) within the nervous system are key issues to be addressed in the future.

Reference:

281. Polynervation and Synapse Elimination by Fiber Type in Neonatal Rabbit Soleus Muscle

James M. Soha, Christopher Yo, David C. Van Essen

The neonatal rabbit soleus is a mixed muscle composed of interspersed fibers of the two major contractile types, fast and slow, in roughly a 2:1 ratio. Immediately after birth, each muscle fiber is innervated by several different motor neurons. A process of synapse elimination during the second week produces the mature pattern of a single neural input to each muscle fiber. We have found that the time course of the elimination of polynervous innervation differs for the two muscle fiber types.

The state of innervation of individual muscle fibers was determined electrophysiologically using intra-
cellular glass microelectrodes and graded nerve stimuli in a partially curarized in vitro preparation. In one series of muscles, fibers identified as singly innervated were labeled by pressure injection of the fluorescent dye Lucifer Yellow. Multiply innervated fibers were labeled exclusively in a second set of muscles. Muscle cross-sections were then stained for alkali-stable ATPase activity to determine the type (fast or slow) of the labeled fibers.

Two age groups were studied. In the younger group (7-8 days postnatal), about 75% of the muscle fibers are multiply innervated, while for the older group (10-11 days postnatal), only about 20% of fibers remain multiply innervated. At the earlier age, we saw no significant difference by fiber type in the level of polynéuronal innervation. At the older age, however, the incidence of multiple innervation was substantially higher in slow compared to fast fiber types. This result contrasts with the suggestion of Riley (1977), based on indirect evidence from neonatal rat soleus, that synapse elimination proceeds somewhat more rapidly for slow fibers.

We have also examined the innervation state at 18-22 days of age, using a cut muscle technique to increase the size of synaptic endplate potentials. Multiply innervated fibers at this age composed about 5% of all fibers examined and may represent a continuing process of neural sprouting and synaptic plasticity. As seen by Taxt et al. (1983), these fibers consistently exhibit one very small endplate potential component along with one much larger. We are now using the Lucifer Yellow labeling technique to determine if this polyinnervation reflects a hypothesized secondary synaptic reorganization (Biology 1984, Abstract No. 237) in which slow motor units expand in size at the expense of fast motor units.

References:

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282. Role of Neuronal and Muscle Activity in Synapse Elimination

Edward Callaway, James M. Soha, David C. Van Essen

At birth, each muscle fiber in the soleus muscle of the bunny receives inputs from two or more motor neurons. During the first two weeks of postnatal development many of these synapses are eliminated, resulting in an adult state in which every muscle fiber is innervated by just one motor neuron. We have focused research on the role of activity, both neuronal and muscle, in regulating neuromuscular synapse elimination. One experiment approaches the question: Do neurons that are active during the period of synapse elimination end up innervating more muscle fibers (i.e., lose fewer synapses) than inactive neurons? A second experiment addresses the role of muscle activity (as distinct from nerve activity) in determining the rate at which synapses are lost.

To test effects of neuronal activity, a small population (5% to 25%) of the neurons innervating the soleus are inactivated for 4 to 5 days during the period of maximal synapse elimination. This is accomplished by insertion of a small tetrodotoxin-laden plug into the one of the two spinal roots that contains the smaller proportion of soleus innervation. Nearly all of the muscle fibers remain innervated by active neurons due to the degree of polyneuronal innervation. After a block beginning at postnatal day 4 and lasting 4 or 5 days, the soleus muscle and its innervation are dissected out of the animal and the twitch tensions resulting from stimulation of individual motor neurons are assayed as an indirect measure of motor unit size. Results to date of 4-day blocks suggest that motor unit size is not greatly affected by neuronal activity, since twitch tensions of active and inactive neurons do not differ significantly. Because there remains a possibility that there is a critical time period for neuronal activity effects that is different than we have tested, experiments blocking neuronal activity for longer time periods and in younger bunnies are in progress.

Preliminary results from experiments blocking muscle activity with alpha-bungarotoxin indicate that decreased muscle activity results in a slower rate of synapse elimination. About 50% of the muscle fibers in two soleus muscles treated from 6 to 11 days postnatally were polynéuronally innervated; this is in contrast to 10% to 20% polynéurononal innervation in normal and control animals of the same age. More animals are being tested to verify these results; older animals will also be treated in a similar manner to determine whether the effect seen is due to sprouting...
of neurons or to greater stability of synapses that would be eliminated in a normally active muscle.

**PUBLICATIONS**


Summary: The identities of most of the molecules involved in neurospecificity and development are unknown. Furthermore, it is unclear how one can identify them. Studies of mRNA complexity indicate that there are thousands of distinct gene products specific for brain. There is a need to explore the range of these molecules. If hybridomas are made, using unpurified brain material, each hybridoma produces a monoclonal antibody (MAb) against only one specific antigen of the set. Our group has isolated a panel of varied MAbs against the *Drosophila* nervous system to use in studying developmental problems (Fujita et al., 1982).

For a simple neural system that is amenable to analysis, we have chosen the developing *Drosophila* eye. The adult eye is a modular structure of some 800 similar ommatidia, each containing only eight photoreceptor neurons of three kinds: R1-6, R7, and R8. They differ in their precise positions, their action spectra, and in the way their axons terminate. R1-6 send axons to the first optic ganglion (the lamina), whereas the axons of R7 and R8 pass through the lamina to synapse in the second optic ganglion (the medulla). This is, in effect, a very simple nervous system of eight neurons of three specificities, reiterated 800 times. Genetic mosaic experiments have shown that the eight cells in each ommatidium are not derived from a single mother cell; they appear to arrive at their specific identities by epigenetic interaction. Development of the cluster occurs in the eye imaginal disc, during the third larval instar, in the wake of a morphogenetic furrow that sweeps from posterior to anterior across the disc. Ahead of the furrow, the cells are unpatterned; behind it, they form into a regular array of clusters of five (R2, 3, 4, 5 and 8). R1, 6 and 7 are added shortly afterward (Ready et al., 1976).

Our group is applying genetic, antibody, and recombinant DNA methodology to identify the molecular specificities of the cell types, to trace from cell-specific antigens to their corresponding genes, and to analyze the molecular events in eye development (Zipursky et al., 1984).

There has been an exciting and unexpected development in the work that has opened up wide possibilities, namely the discovery that a large fraction of the MAbs against the *Drosophila* nervous system also cross react with various components of the human nervous system (Miller and Benzer, 1983). From the point of view of human brain structure alone, the availability of a large panel of cell-specific markers adds a new dimension to brain anatomy. Much of the mapping with antibodies in the past has been directed to known peptides or neural transmitters. These MAbs extend the range to unknown molecules, providing new markers for anatomical structures, cell types, and antigen molecules. These experiments are being done in collaboration with Dr. Carol Miller, who is a Visiting Associate at Caltech and, as Chief of Neuropathology at the University of Southern California Medical School, has ready access to human brain material. Dr. David Hinton is a postdoctoral fellow who works between both laboratories. There are several questions that these cross-species investigations can approach. Are there clusters of genes that control the expression of groups of nervous system molecules, and do such clusters tend to remain together in evolution? Do new nervous system-specific molecules appear, or does evolution consist largely of modifying much the same set? Are conserved molecules used in the same fashion in the various nervous systems? There is much to explore in the unknown molecular landscape of the nervous system to provide the necessary ground work for tracing its development from the genome.
References:

283. From Monoclonal Antibody to Gene for a Neuron-specific Glycoprotein

Tadmiri R. Venkatesh, Stephen L. Zipursky

MAb 24B10 was isolated as described by Fujita et al. (1982) from mice immunized with homogenates of adult retina. It stained adult photoreceptor cells and their axons. A developmental study (Zipursky et al., 1984) that the antigen recognized by MAb24B10 (Ag24B10) appears at an early stage of cellular differentiation. The early expression of this antigen and its localization to the cell body and axon suggest that this antigen may play an important role in photoreceptor cell development. To approach the question of its function, we have characterized the antigen, cloned the gene encoding it, and initiated genetic analysis.

Ag24B10, purified by immunoaffinity chromatography, was shown to be a 160 kd glycoprotein which comprises 0.8% of the total protein in the adult retina. Using radiolabeled-Concanavalin A (ConA) and SDS-polyacrylamide gel electrophoresis, the 160 kd species was shown to be one of three adult retina-specific ConA-binding proteins. It is also one of about ten major ConA-binding proteins in the developing eye disc. In addition to binding ConA, indicating the presence of D-glucosyl and/or D-mannosyl residues, Ag24B10 bound ricinus lectin, indicating the presence of D-galactosyl residues. Dr. David Teplow has determined 19 of the first 21 amino acids at the N-terminus of Ag24B10. Using this information, Dr. Suzanna Horvath synthesized a population of oligonucleotide probes that could encode a selected region of the N terminus. These probes were used to screen a Drosophila genomic DNA library.

A single DNA clone was identified, the sequence of which matched the N terminus of the amino acid sequence. This segment of Drosophila DNA contained the only sequences in the Drosophila genome that bound to the oligonucleotide probes, as determined by Southern blots of the cloned and genomic DNA. The labeled DNA clone recognized a 4.2 kb poly(A) head-specific RNA. This is of sufficient size to encode a 160 kd polypeptide (Zipursky et al., 1985).

The Ag24B10 gene maps to the 100B3-100B9 region on the right arm of chromosome 3, as determined by in situ hybridization. Using duplication 48, in which a segment of 3R from 100B8-100B9 to the tip has been translocated to the tip of the short arm of the X (constructed by Dr. Ross MacIntyre of Cornell University and kindly provided by Dr. John Merriam of UCLA), the Ag24B10 gene was shown to hybridize at a location distal to the break point. These data localize the gene to the 100B8-B9 region. Experiments are in progress to identify deficiencies removing the 24B10 gene which will facilitate the isolation of mutations.

References:

284. Disruption of the Developing Photoreceptor Array by a Drosophila Eye Mutation

Patricia J. Renfranz

The precise neural pattern of the compound eye of Drosophila arises in the eye-antennal imaginal disc during the third larval instar. One approach towards elucidating the molecular mechanisms controlling eye development is the analysis of mutations that disrupt pattern formation. In order to find which of these affect the initial developmental events occurring in the eye disc, monoclonal antibodies which highlight specific pattern elements of the developing eye (Zipursky et al., 1984) were used to screen the eye discs of various mutants. Most of the mutants showed normal arrays in the eye disc, indicating that their mutations act at a later stage of development. However, a few mutations seem to affect initial formation of the array.

The mutation Rough Eye (Roi), a homozygous lethal on the second chromosome, appears to disrupt the precise clustering of eight photoreceptor cells into ommatidia. Clusters in Roi eye discs have abnormal shapes and sizes, perturbing the orderly wild-type array. The adult eyes of Roi flies are of normal size;
tangential sections reveal ommatidia composed of anywhere from four to ten photoreceptor cells. It can therefore be inferred that the irregularity of the array seen in the eye disc may result from variable and inappropriate numbers of photoreceptor cells joining each cluster.

The Roi mutation arose spontaneously on a second chromosome carrying various inversions, as well as other mutations. In a series of crosses designed by Dr. Loring Craymer, involving levosynaptic and dextrosynaptic chromosomes (see Craymer, 1981), Roi is currently being recombined onto an otherwise normal chromosome. Once genetic work on this mutation is completed, molecular studies can be undertaken in order to understand the influence of this gene upon eye development. Since Drosophila eye development involves non-clonal regulative mechanisms, the Roi gene product could be involved with cell-cell contacts, or other events important in neural pattern formation.

References:


Utpal Banerjee

The crystalline precision of the Drosophila compound eye is a result of well over a hundred genes working in proper synchrony. Malfunction of these genes can cause perturbations to the pattern of ommatidial arrangement or neuronal connectivity. The mutant sevenless (sev) lacks a single neuron, photoreceptor cell 7, in each facet of the eye. The specific action of the gene made us choose this mutant for detailed investigation.

The sev mutation has previously been mapped to the 10A1-2 bands of the X chromosome (Harris et al., 1976). We are currently trying to clone this gene. First, a rapid "deep pseudopupil" (Franceschini and Kirschfeld, 1971) screening method was devised to look for new alleles of sev. Sevenless flies lack a central cell in each ommatidium, hence the image from the overlapping patterns is distinguishable from that in wild-type flies. Also, in a color-choice behavioral screen, sevenless flies, lacking the UV photoreceptor, choose visible light over ultraviolet, unlike wild-type flies which overwhelmingly prefer UV. Using a combination of these two tests, three P factor-induced mutants were isolated by screening 23,500 male progeny from dysgenic crosses. Another 30,000 EMS-treated flies were tested, yielding eight new mutant alleles.

Chromosome squashes of the P-allele sevP1 were hybridized in situ to P-element DNA. Approximately 50 P-insertion sites were seen per genome. A genomic library of DNA from sevP1 was constructed in the EMBL3 vector system. Screening this library with P-element DNA identified a large number of clones of which one maps at the right edge of the vermilion band at 10A1 on the X chromosome. We are currently investigating the possibility that this clone is from the sevenless complementation group.

References:

286. Monoclonal Antibody Staining of Drosophila Embryos

John A. Pollock

To observe the embryonic development of the nervous system, a collection of 164 monoclonal antibodies (MAbs) has been screened on whole mounts of embryos (12-14 hours after fertilization), following the method of Mitchison and Sedat (1983). A number of other interesting staining patterns were observed. Several antibodies stained specific surface structures, notably MAb3B4 (see front cover photograph), which brilliantly stained the interdigitating setal belts of the thoracic and abdominal segments and the three pairs of Keilin's organs. In another class were MAbs that stained the nervous system, or regions of it. For example, MAb21A6 stained just the small sense rods of the scolopophorus organ (chordotonal organ) which is located between the body wall and the muscles of each segment (Hertwark, 1931) and other surface sensory organs. MAb22C10 stained these rods plus their enveloping cells, the basal sensory cells, and the sensory neurons of the scolopophorus organ. MAb22C10 also stains other sensory neurons and cells of the nervous system, in a temporally regulated manner.

Another nervous system-specific antibody, MAb11F9, stained cells in the ventral nerve cord and brain. In a preliminary study of the time course of
staining, we have observed that at -14 hours after fertilization, only one pair of cells was stained in each segment of the ventral nerve cord. With time, other symmetrically paired cells become stained. By late embryogenesis, a larger number of cells appeared in the brain. By the time of hatching, nerve tracts could be seen in the brain and the ventral ganglion.

MAb3E1 specifically stained the developing tracheal system. Between six and eight hours after fertilization, the branches of the two longitudinal trunks, which eventually connect the anterior and posterior spiracles, could be seen as pairs of fine lateral lines in each segment. Other MAbs specifically identified muscles or nuclei.

Of particular interest in our study of the *Drosophila* retina is the embryonic development of the larval photoreceptor system (Bolwig’s organ). Neurons from Bolwig’s organ project through the eye-antenna imaginal disc to the larval brain. The axons of prospective adult developing retinal cell axons also project through the optic stalk nerve into the visual formation centers. If Bolwig’s nerve serves as a guide, disruption of embryonic development could affect the organization of the adult eye and its wiring to the brain. In an attempt to cause defects in development of Bolwig’s organ, MAb24Bl0 and other MAbs, which stain Bolwig’s organ and nerve in the larva, are being microinjected into preblastula embryos (up to 2.5 hours after fertilization). A second method for inducing phenocopy mutations of Ag24Bl0 that may affect the embryonic development of Bolwig’s organ employs the microinjection of antisense Ag24Bl0 mRNA (Rosenberg et al., 1983).

Some possible results are 1) no effect, 2) delay in the development of either the organ or its axonal projections, but normal development of larval and adult visual systems, or 3) gross disruption of the development of Bolwig’s organ such that no larval photosensory system is present. If result (3) is obtained, it would be of interest to see whether the adult eye wiring develops normally without the need for Bolwig’s nerve as a guide.

References:

287. Generation of Monoclonal Antibodies to the Developing *Drosophila* Visual System

John A. Pollock

We are extending the number of monoclonal antibodies (MAbs) with which to study the *Drosophila* retina. Originally, 148 MAbS (Fujita et al., 1982) were generated by immunizing mice with *Drosophila* brain, head, or retina homogenates. In generating new MAbs, two different preparations have been employed. First, a modification of Paulsen’s (1984) method of isolating blowfly rhabdomes has been used to enrich rhabdomere bundles from *Drosophila*. Freshly excised *Drosophila* eyes were placed in distilled water at 4°C and gently broken up. This was briefly centrifuged to separate floating debris (tracheae, fat, and basement membrane) from rhabdomes, cone and pigment cells, and cellular organelles. The resuspended pellet was then centrifuged in a 25% Percoll gradient. Rhabdomere bundles formed a broad band in the middle of the tube and were easily identified by microscopic inspection of the fractionated gradient. Treatment with EGTA opened the bundles, which were then washed by repeated centrifugation in phosphate buffer and homogenized prior to injection into the mouse. Three such injections were performed over a two-month period. After fusion to mouse myeloma cells, ten MAb-producing cell lines were cloned out of ~250 tested on cryostat sections of fly heads. Three MAbs were retina-specific, similar to MAb24Bl0 (Zipursky et al., 1984, 1985). Two MAbs stained the intrareticular space, two stained hypoderm, and one each stained cone cells, pigment cells, or nuclei.

The second fusion experiment (in progress) combines the principle of immunosuppression described by Matthew and Patterson (1983) with the injection of isolated imaginal discs as the immunogen. This technique involves initially immunizing the mouse with a tissue preparation that lacks a particular substance of interest. Activated lymphocytes are then suppressed by the injection of the immunosuppressive drug, cyclophosphamide. When the immune response is sufficiently suppressed to the primary immunogen, the animal is given a second dose with an immunogen in which the substance of interest is present. For the purpose of this experiment, we have employed the Eugene et al. (1979) method for isolating thousands of imaginal discs from third instar larvae of *Drosophila*. 
The procedure isolates all the various imaginal discs in a fraction that is essentially free of other larval body parts. Using a 45% Percoll gradient, a preparation deficient in eye discs was prepared. Thus, the initial immunization was done with this deficient disc preparation, followed by injections with cyclophosphamide, the final booster injection being with material enriched for eye discs. It is hoped to acquire MAb-producing cell lines that specifically identify molecules unique to the developing eye imaginal disc. These experiments have been carried out with the assistance of To Ha Thai of our Division's Monoclonal Antibody Facility.

References:

288. Isolation of Genes Encoding Antigens Expressed During Eye Development

Dennis G. Ballinger

The antigen recognized by MAb 22C10 (Ag 22C10) appears very early during the development of the photoreceptor cells in the Drosophila larval eye imaginal disc (Fujita et al., 1982; Zipursky et al., 1984). The MAB also stains a small subset of the developing neurons present in the ventral nerve cord of early embryos and a larger set of adult neurons and their axons. On Western transfers of NP-40 solubilized head homogenates, Ag 22C10 appears as a broad band of molecular weight 160,000-200,000. Immunoaffinity purification of Ag 22C10 from adult fly heads yields a protein complex that does not enter the separating gel after boiling in the presence of sulfhydryl reducing agents and SDS. This complex was used as an immunogen in mice to produce a series of new MAb directed against components of the purified antigen. Among antibody-producing hybridoma lines, 50 showed staining on cryostat sections of fly heads. Of those, 29 produced MAb with a staining pattern similar to MAb 22C10. These results indicate that the immuno-
screening. They will next be screened by in situ hybridization to tissue sections, to identify their precise temporal and spatial patterns of expression. Any potentially interesting genes can be mapped on polytene chromosomes and further studied by inducing and characterizing mutations in them.

A similar approach is being used to find gene products associated with a specific cell type. In flies homozygous for the eye mutation sevenless, one specific photoreceptor cell (R7) is missing from each ommatidium. Poly(A)+ RNA preparations from wild-type and sevenless heads are being used to screen a Drosophila genomic DNA library. Several sequences that appear to be represented in the wild-type mRNA but not the mutant mRNA have been identified. Further retesting and analysis should provide genes that are expressed differentially by wild-type and sevenless flies. These genes may include the sev gene itself, the gene encoding an R7-specific opsin, and genes encoding molecules involved in the specific synaptic connections formed by R7. These genes may provide R7-specific markers and contribute to the study of the regulation of cell type and synaptic specificity during eye development.

290. Antigenic Homologies Between Mammalian and Drosophila Retina

David R. Hinton, Carol A. Miller

Monoclonal antibodies (MAbs) may be used to visualize, identify, and purify specific polypeptides which show cellular specificity in the nervous system. Recently, we have demonstrated that a large fraction of MAbs made with Drosophila nervous system as primary immunogen also cross react with selective components of the human nervous system (Miller and Benzer, 1983). While it is difficult to make specific regional comparisons between Drosophila and vertebrate brains, the retina is relatively conserved in structure among the vertebrates and approximate comparisons to fly retinal structures are possible. In collaboration with Janet Blanks of the Doheny Eye Institute, we have chosen retina as a model system for studying antigenic conservation in the nervous system (Blanks et al., 1984).

Approximately 45% of MAbs that react with various regions of Drosophila eye and brain cross react with vertebrate retina. Five patterns of specificity emerge: cell nuclei, synaptic regions, neuronal membranes, photoreceptor cells and Müller cells. The photoreceptor cell can be antigenically dissected into several subcellular regions, including cell body, inner segment, and outer segment. A high degree of topographic homology is observed among the vertebrates and some of the MAbs also react with comparable regions in Drosophila eye. One of these antigens, 8C3, has been purified from both human and Drosophila and shows similar migration pattern on polyacrylamide gels. We are now further characterizing several of these antigens for peptide and amino acid sequence homologies.

A few known molecules such as opsin have been demonstrated to be conserved in Drosophila and some vertebrates. Our MAb panel extends these studies to a variety of unknown eye-specific molecules. If these antigens are conserved through evolution, they may prove to be functionally important in the nervous system.

References:

PUBLICATIONS
DEVELOPMENTAL GENETICS

Edward B. Lewis
Summary: Our group is primarily studying how genes control development. We use as a model system a giant cluster of genes that controls much of the basic body plan of *Drosophila*. This cluster, or bithorax complex (BX-C) as it is called, appears to be directly responsible for directing the remarkable degree of specialization that characterizes the abdominal region of the fly as well as part of the thoracic region. For example, certain mutants within this gene complex result in flies with eight legs instead of six, or four wings instead of two. We maintain close collaboration with three other groups who are working on various aspects of this model system—groups headed by Dr. Welcome Bender at Harvard Medical School, Dr. Ian Duncan at Washington University, and Dr. David Hogness at Stanford University Medical School.

Since our last report a major development in the field of genetic control of development has been the independent discovery of homeoboxes by two groups (McGuinness et al., 1984; Scott and Weiner, 1984). The homeobox is a highly conserved sequence of amino acids within proteins coded for by genes of the BX-C and a related complex in which the original homeobox was first found, namely, the Antennapedia-complex (ANT-C). The latter gene cluster, extensively studied by Dr. T. Kaufmann and colleagues at the University of Indiana, has many similarities to the BX-C except that it controls segmental differentiation in the anterior region of the body, while the BX-C controls it in the posterior half. Genes in these complexes have presumably arisen by a process of tandem duplication followed by gradual divergence in function. The homeoboxes appear to provide evidence for just such an evolutionary process. Not only are the DNA sequences that code for the homeoboxes very similar within each complex, the ANT-C and BX-C homeobox sequences are remarkably similar to each other, suggesting that at least several genes in the two complexes have had a common ancestral origin. The analysis of the BX-C homeoboxes has been made possible by the molecular mapping of the complex by Dr. Welcome Bender and has shown that they occur in three regions of the complex: one in the Ultrabithorax (Ubx) gene and two in the DNA domain directing differentiation of the abdominal regions, namely, the *infra-abdominal-2* and *infra-abdominal-7* regions of the complex. The precise role that such homeoboxes play in development is not clear but one guess is that they are regions that bind specific DNA sequences in other genes thereby turning such genes off or on.

References:

291. The Bithorax Complex Controls the Initiation of Gonads

Rollin H. Baker, E. B. Lewis

The formation of gonads in higher organisms is confined to the abdominal regions of the body. In the fly they are found in the fifth abdominal segment. The actual origin of the gonad however may involve several segments if the fly follows the same pathway of organization of gonadal tissue as found in more primitive insects. Thus, in the latter case, gonadal primordia from several segments are recruited to form the gonad itself. (It is known that in human beings gonads arise from specialized ducts that may have a segmental origin, although the precise way in which body segments in flies and persons correspond to one another is controversial.)

We have been fortunate in being able to find within the BX-C recessive mutants that cause total loss of gonads in males and females. Since the primary effects of such mutants appear to be to prevent the animal from developing its fourth abdominal segment properly, we call them *infra-abdominal-4* mutants (*iab-4*). Such mutants are easily perpetuated by keeping them heterozygous; that is, flies with one normal chromosome and one containing the mutant are fully fertile and true breeding strains can be established that will produce normal and mutant flies
in the ratio of 2:1. (A trick is used to prevent the normal chromosome from displacing the mutant chromosome by introducing a recessive lethal gene, as well as crossover suppressors, into that chromosome; animals that derive such a chromosome from both parents die, thereby ensuring that the mutant chromosome will not be lost from the population.)

At first the effects of the BX-C genes on development could only be readily studied in the cuticular structures or tracheal system of the animal, tissues that are of epidermal origin. However, studies by Jan and Jan (1981), Lawrence and Johnston (1984) and Akam and Martinez-Arias (1985) have shown that muscles are also affected by genes of the BX-C, thus showing that mesoderm as well as ectodermal tissues are affected. Since the gonad is also of mesodermal origin, our results extend the range of action of the BX-C genes to another major tissue of mesodermal origin.

We have now found two cases in which inactivation of a gene known as infra-abdominal-5 (iai-5), located in the chromosome immediately to the right of iab-4, causes overexpression of the normal iab-4 gene in the sense that certain parts of the cuticle of the third abdominal segment (namely, parts that differ between the third and fourth segments) begin to transform towards the corresponding parts of the fourth segment. This latter effect represents a dominant gain of function in contrast to the recessive loss of function that occurs in the case of the iab-4 mutants. We are now attempting to determine whether this effect extends to the gonad as well as to cuticular structures.

References:

292. The Search for Cryptic Mutations in the Bithorax Complex

E. B. Lewis, Rollin H. Baker, Josephine Macenka

We are currently using a genetic screen that allows us to detect chromosomal rearrangements that have one breakage point with the BX-C and another elsewhere in the genome. Such rearrangements disrupt the somatic pairing that normally characterizes the chromosomes of Drosophila and other flies. As a result there is a change in the expression of certain mutant genes that enables us to detect such rearrangements in the first generation following irradiation of adult males with X rays. The method essentially can detect all breakages that occur within the complex, even cryptic ones that would have no direct effect on any gene within the complex. Two rearrangements have been uncovered thus far that at first sight seemed to be of this cryptic type. However, careful study has shown that they have a barely detectable recessive loss of function in the bithoraxoid (bxd) gene, strong mutants of which produce eight-legged flies and cause profound modifications in certain sense organs of the larva. In order to locate the breakage within the bxd domain, one of these semi-cryptic mutants has been combined with a virtually identical rearrangement except for having a breakage point immediately to the left of the complex; in this way, a synthetic deletion has been found that on the basis of embryonic morphology is deleted for the bulk of the bxd function as well as the Ubx function. Confirmatory evidence of a breakage within the bxd domain has been obtained by Dr. W. Bender and colleagues (personal communication). As yet we have no convincing evidence that the BX-C can be split, but there may still exist regions within the complex that when separated from one another might still function normally. However, since the BX-C has remained intact over presumably a long evolutionary period judging by the homeobox evidence cited above, there may well be some subtle functional requirement for the right half of the complex to be in close proximity to the left half.

293. Transabdominal: Misregulation within the Bithorax Complex

Susan E. Celniker, John Butman¹, David J. Baker

The mutant Transabdominal (Tab) is a dominant gain-of-function mutant that puts stripes of abdominal tissue on the notum. As previously reported, the dominant phenotype of Tab is produced by the juxtaposition of sequences from 90D next to an abdominal gene of the BX-C (Biology 1984, Abstract No. 247). To understand this misregulation of an abdominal BX-C gene, we have induced revertants of Tab with X rays.
Using cloned BX-C DNA (obtained from F. Karch and W. Bender, Harvard University) from the distal end of the complex, we have begun to analyze Tab and ten revertants to locate the specific changes that have occurred at the DNA level. Since Tab and the revertants are lethal when homozygous and lethal when over a deficiency for the BX-C (Df-P9), DNA for genomic blot hybridization was made from flies carrying the Tab chromosome and the original chromosome that Tab was induced upon. The Tab breakpoint has been localized on the DNA map of the BX-C to +183 (F. Karch and W. Bender, personal communication); this represents the most distal break of those analyzed in the complex. Seven of the revertants are translocations and the breakpoints for five of them have been identified by Southern analysis. The revertant breaks span over 15 kb of DNA, though all map as predicted by cytological analysis to the left of the Tab breakpoint. Thus, reversion of the Tab phenotype occurs when the proximal end of the inversion is further disrupted by chromosomal rearrangement.

Using the DNA restriction fragment that spans the Tab breakpoint, we have obtained DNA from the proximal breakpoint from a recombinant Tab library. By restriction enzyme digest mapping, we found that the recombinant phage has 7 kb of DNA from the 90D region which may include the new cis regulatory region responsible for the misregulation of the A6 or A7 abdominal BX-C gene. Further investigation will involve sequencing the DNA of the Tab rearrangement and an analysis of the transcripts from the wild-type and Tab regions.

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294. A Genetic Analysis of the Pairing Rules Affecting the Interaction of the zeste and white Genes

Sarah M. Smolik-Utlaut

In the past year I have continued a genetic analysis of the critical region necessary for the pairing of transvection-sensitive genes in Drosophila. Transvection is a type of complementation of gene function that is influenced by the extent of the somatic pairing of homologous chromosomes. Rearrangements that disrupt the pairing of the homologues tend to decrease complementation. There are three examples whose gene expression depends upon the synopsis of the homologous chromosomes carrying them: the bithorax complex (BX-C) (Lewis, 1954), the decapentaplegic complex (DPP-C) (Gelbart 1982) and the interaction between the zeste and white loci (Jack and Judd, 1979).

Only those rearrangements having breakpoints within a "critical region" proximal to the synapsis-dependent gene function can disrupt transvection. The proximal-most endpoint of the DPP-C critical region appears to be coincident with a constriction on the polytene chromosome.

The results of the experiments I have performed suggest that 1) a critical region as it is defined for transvection at BX-C and DPP-C does not exist for the zeste-white interaction, 2) the constriction at 3C is not by itself necessary for the synapsis-dependent expression of the white locus, and 3) the zeste-white interaction is less sensitive to the disruption of pairing than transvection at DPP-C and BX-C.

The interaction of the zeste (z) (3A3) and white (w) (3C2-3) loci is transvection sensitive. Female flies homozygous for the z1 mutation have lemon yellow eyes while males have a wild-type eye color. If the females have unpaired w+ alleles, they also have wild-type eyes. Rearrangements that restore pairing of the w+ alleles also restore the lemon yellow eye color.

To ask which breakpoints would disrupt the z phenotype and thus define the critical region for the zeste-white interaction, z1 males were irradiated, mated to z1 females and the F1 progeny screened for females with red eyes. A number of transvection-disrupting rearrangements were generated and it was found by an analysis of cytological and in situ chromosomal squashes that most of the breakpoints fell between the w and Notch (3C7) loci. This demonstrates that the critical region for w is small and suggests that the constriction at 3C may act as a boundary for it. To test this hypothesis I have tried to expand the critical region by deleting the constriction. I irradiated z1 males and mated them to z1 females that were deficient for the constriction. The analysis of the zeste-suppressing rearrangements led to a surprising result. Genetically, it appears that the critical region has been expanded; that is, half of the rearrangements only suppressed z when over the constriction-deficient chromosome. However, all of the breakpoints are in 3C. If the constriictions are true
boundaries, then some of the breakpoints should have been between 3C and the next proximal constriction at 7B.

To ask if the pairing rules for the *zeste-white* interaction are really different from those for DPP-C and BX-C, I am looking at insertions of *w*⁺ (as translocations or P element insertions) into the critical regions of DPP-C and BX-C. If the *w* locus can respond to the transvection rules of these loci, then breakpoints between the insert and the centromere will disrupt the *z* phenotype. If the pairing rules are the same as those found on X, then the *z* phenotype will be suppressed only if the breakpoint is very close to the insertion.

References:

295. Effects of Heat Shock on *Drosophila* Embryos

Susan J. Eberlein

Heat shock of pre-adult *Drosophila* causes developmental defects (Mitchell and Lipps, 1978). These defects probably result from disruption of normal gene expression via the heat shock response. The heat shock response includes reduction of normal mRNA and protein synthesis and induction of several "heat shock proteins" (Tissieres et al., 1974). This study uses heat shock to study stage-specific processes in early development. Short, intense shocks (2-3 min, 42-43°C) are administered to carefully staged embryos within the first five hours of development. The shocks induce the heat shock response in syncitial blastoderm (2.5 hours old) and later stages, but do not alter protein synthesis pattern in pre-blastoderm eggs, which survive a three-minute shock response less well than older eggs. The ability of older eggs to induce the heat shock response probably enhances survival. Two classes of defects in embryonic cuticle pattern are observed following shock. General defects occurring after shock at any stage resemble non-specific tissue damage. Stage-specific defects are noteworthy. Abnormal segmentation follows shock at higher blastoderm or one hour after the beginning of gastrulation. Defects resembling pair rule mutations, which delete parts of alternate segments (Nusslein-Volhard and Wieschaus, 1980), occur at high frequency. Animals shocked during early gastrulation (4 hours old), a stage between these two sensitive stages, do not exhibit segmentation defects. Instead, defective germ band shortening commonly results. (Germ band shortening normally occurs about five hours after gastrulation.) Development does not simply stop at the time of the disrupted process; later events such as cuticle formation still take place. This gives the impression that a specific process in the developmental sequence is omitted or altered. Stage-specific defects do not occur following pre-blastoderm shock, suggesting involvement of the heat shock response in causing these defects. The mutant *hairy* was also investigated. Extreme alleles show a striking pair rule phenotype (Nusslein-Volhard and Wieschaus, 1980). Heat shock of *hairy* heterozygotes or homozygotes at blastoderm specifically increases the frequency of the extreme *hairy* phenotype.

References:

296. The Transmissibility of Long-armed Chromosomes

*Loring G. Craymer III*

In *Drosophila*, chromosomes with exceptionally long arms tend to cause the death of individuals carrying them, as I noted in a previous report (Biology 1983, Abstract No. 217). The lethality is somewhat temperature sensitive: I have been able to recover some chromosomes at 18°C that cause lethality at 25°C, although there are other chromosomes that cause lethality at both temperatures. The lethality appears to be directly related to arm length. Below a critical length, no effects are observed on either viability or phenotype. As arm length increases beyond this point, viability decreases and survivors show signs of generalized cell death. The phenotypic abnormalities become increasingly severe as arm length is increased to a point at which the long-armed chromosome results in complete lethality.

This phenomenon has important consequences for chromosomal evolution. With an upper limit on arm lengths, chromosome lengths are necessarily limited.
Except for organisms with small genomes, multiple chromosomes should be a necessity to avoid overstepping the limit on maximal arm length.

The standard *Drosophila* karyotype includes five major chromosome arms of approximately equal size. The primitive karyotype includes each of these arms as a separate chromosome, but the more advanced species tend to have two pairs of these arms attached to form two large metacentric chromosomes. I know of no *Drosophila* species with Robertsonian fusions of the major arms—cases where two of the major arms are attached to form one chromosome arm with twice the major arm length—although there are cases where a long-armed chromosome is the result of Robertsonian fusion of one of the major arms with an arm of the Y chromosome. This suggests that the critical arm length may be less than twice the major arm length in *Drosophila*. It seemed worthwhile to investigate this point experimentally.

To determine the critical length, a more reliable criterion was needed than either viability or incidence of phenotypic abnormalities. Fortunately, the chromosomes that could be recovered but caused drastically reduced viability also seemed to be poorly transmitted through the male. For several reasons—viability differences among them—it was not possible to reliably compare the transmission rates of different chromosomes. However, since viability was known to be affected by temperature, it seemed reasonable to expect that transmissibility would also be affected by temperature, and an experiment was designed accordingly.

A series of long-armed chromosomes was tested which did not noticeably affect either viability or phenotype. Males carrying a chromosome to be tested were raised at either 18°, 25°, or 28°C. Females from a tester stock were raised at 25°C. Matings were made and the males discarded after mating. Progeny were grown at 25°C. This scheme avoids any differential viabilities between the different crosses and ensures that each experiment measures only transmissibility through the male of the long-armed chromosome.

The transmissibility of two of the tested long-armed chromosomes proved to be affected by temperature, and the direction of the effects was as predicted: the long-armed chromosomes transmitted more poorly at the higher temperatures. For one of these chromosomes, the long arm clearly spans less than two major arm lengths.

**PUBLICATIONS**


THE FOLLOWING REPORTS ARE BY GRADUATE STUDENTS IN THE DIVISION OF BIOLOGY WHO ELECTED TO DO THEIR THESIS WORK IN THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

297. Molecular Analysis of a Cis-Acting Negative Regulatory Element in Drosophila melanogaster

William Mattox, Norman Davidson

Support: National Institutes of Health, USPHS

Mutations at the Beadex locus of Drosophila melanogaster result in a characteristic loss of tissue along the wing borders (wing scalloping). Genetic analysis of these mutations has revealed that they cause an increase in the activity of the closely linked heldup-a gene (Lifshytz and Green, 1979). Loss of function mutations at the heldup-a gene itself cause flies to hold their wings in an upright position. These mutations strongly suppress the cis Beadex allele. These results have led to the proposal that the Beadex locus normally functions as a cis-acting negative regulatory element for the heldup-a gene. We are testing this proposal by investigating the molecular characteristics of the Beadex-heldup-a complex in both mutant and normal flies.

Using a transposon-tagging approach, we isolated recombinant phage clones containing DNA sequences encoded at the Beadex locus. Analysis of a number of Beadex alleles and one heldup-a allele indicates that the Beadex locus is confined to a small region less than 1 kilobase (kb) in length located very near, if not adjacent to, the heldup-a gene. Analysis of mRNAs encoded in this region has identified only two rare transcripts. One transcript is 2 kb in length and makes up approximately $2 \times 10^{-5}$ of polyadenylated RNA in 0-4 hr embryos. This transcript has not been detected in later stages of development. However, another transcript, which is 4 kb in length, becomes detectable 4-8 hr after fertilization and persists until eclosion. This 4 kb transcript makes up no more than $1 \times 10^{-5}$ of polyadenylated RNA at any time during development.

Structural analyses of these transcripts make it clear that they contain overlapping sequences and that the genetically defined region corresponding to the Beadex locus lies within them, probably in an intron. We are currently investigating the effects of Beadex and heldup-a mutations on these transcripts.

Reference:

298. A Homologue to a Retroviral Reverse Transcriptase Gene: In Search of its Origin and Function

Lei Yu, Robert J. LaPolla, Norman Davidson

Support: California Foundation for Biochemical Research

A clone, RT26A, was isolated from the cDNA library of membrane-associated polysomal polyadenylated RNA from a myogenic mouse cell line, BC3H-1 (LaPolla et al., 1984). The probe used in screening the library was a cDNA clone for the $\beta$-subunit message of the acetylcholine receptor from the electric ray Torpedo. This mouse cDNA clone was analyzed by the dideoxy nucleotide chain termination sequencing method and was found to be homologous to the 3' half of the pol gene of Akv, a murine retrovirus. Akv is a murine leukemia virus and is associated with a high incidence of T-cell leukemia in AKR mice. It is endogenous to many laboratory mouse strains and cell lines and its provirus is spontaneously induced in the animal before birth. The virus production is continuous during the life of the AKR mice and the virus produced is ecotropic, that is, it can infect mouse cells.

The cDNA clone we isolated is 1770 base pairs in length, has an open reading frame covering the entire region, and has a stretch of more than 100 adenosine residues attached to it. It overlaps with the Akv pol gene in the region from the middle to the 3' end of the gene and shares 97% homology with it. Since a viral RNA transcript terminating at the end of the pol gene has never been reported, a question is raised as to the origin of the messenger RNA from which RT26A was derived. Is it the product of an endogenous virus, a pseudo-viral sequence as the result of recombinational events, or a cellular reverse transcriptase gene? What does it do in the cell? An investigation is under way to address these questions.

Reference:

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299. Molecular Biology of the Acetylcholine Receptor
Gamma Subunit from Mouse
Lei Yu, Robert J. LaPolla¹, Norman Davidson¹
Support: California Foundation for Biochemical Research
National Institutes of Health, USPHS

The nicotinic acetylcholine receptor (AChR) is a postsynaptic integral membrane protein complex that plays an essential role in muscle contraction by opening its ion channel upon the binding of acetylcholine molecules. The receptor is composed of four subunits, α, β, γ, and δ.

Our goal was to isolate the cDNA sequence for the AChR γ mRNA molecules in mouse, so that its structure and function could be studied. Using the cDNA clone for the AChR gamma subunit of Torpedo as hybridization probe, several cDNA clones were isolated from the cDNA library of a mouse myogenic cell line BC3H-1. These cDNA clones belong to two overlapping groups and have different restriction endonuclease recognition patterns. Sequence analysis of the representative clones from both groups was performed. One clone, designated MARG9, contains the entire coding region for the mouse AChR γ subunit precursor plus part of the 5' untranslated region and the complete 3' untranslated region. The sequence comparison between the mouse AChR γ subunit and the ones published for human and calf shows a strong homology, being 90% at the protein sequence level in both cases. The other clone, designated MARGO, appears to be a copy of part of the unprocessed primary transcript of AChR γ gene. It contains part of the AChR γ protein coding sequence interrupted by an intervening sequence and flanked at its 3' side by another intron. The intron positions are exactly the same as those reported for the gene for the human AChR γ subunit.

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300. The Molecular Biology of the Acetylcholine Receptor
Katharine Mixter Mayne, Michael M. White, Henry A. Lester, Norman Davidson¹
Support: National Institutes of Health, USPHS

The electrophys of Torpedo has been used for some time as a rich source of acetylcholine receptor for biochemical investigation. Work in a variety of laboratories has shown that the receptor is an integral membrane protein complex of four different polypeptides, α, β, γ, and δ, in a 2:1:1:1 stoichiometric ratio (Raftery et al., 1980). These four subunits have been cloned as cDNA copies of their messages in this laboratory and by others (Claudio et al., 1983; Hershey et al., 1983; Numa et al., 1983).

We have recently reported the cloning of a cDNA encoding the δ subunit of the mouse acetylcholine receptor. This clone was isolated from a murine myogenic cell line by hybridization to the Torpedo γ subunit cDNA. We designated it as a δ because, at the amino acid level, it shows greater homology to the Torpedo δ subunit (La Polla et al., 1984).

As a more functional criterion for assigning the identity of this subunit, we decided to try to incorporate the mouse δ into the Torpedo complex in place of the Torpedo δ. We used the SP6 transcription system to synthesize mRNA for each of the Torpedo subunits as well as the mouse δ. When all four Torpedo subunit RNAs are injected into Xenopus oocytes, a large response to acetylcholine is detected in a voltage clamp assay. When the Torpedo β or γ RNAs are omitted, no detectable response to acetylcholine is seen, whereas a small (3%) response is detectable without the Torpedo δ. When the Torpedo δ RNA is replaced with the mouse δ mRNA a 3-4 fold greater response is measured than that detected with a complex of the four Torpedo subunits. If mouse δ RNA is used in place of the Torpedo γ subunit RNA, no response is measured. These results unequivocally identify the mouse subunit as a δ. These results have been recently published (White et al., 1983).

Our most recent work has been to address the possibility that a discrete region of the subunit contains the structure(s) that defines it as a δ. All four subunits are homologous in sequence and predicted secondary structure. It is not obvious, even after close examination, where this region might be.

We intend to construct hybrid cDNA molecules between the δ and the γ molecules that will fuse the δ and γ proteins. These hybrids will be tested in the Xenopus microinjection assay to see if they can act as a δ or as a γ. A positive result would localize the subunit-specific region to as small a portion of that subunit as was included in the hybrid.
References:
Hershey, N. D., Noonan, D. J., Mixter, K. S., Claudio, T. and Davidson, N. (1983) *Cold Spring Harbor Symp. Quant. Biol.* XLVIII, 79-82. (See also several other related papers in this volume.)

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Cell Sorting Facility

**Supervisor:** Ellen Rothenberg  
**Staff:** Patrick F. Koen, Rochelle A. Diamond (Sailor)

The Biology Division's Flow Cytometry/Cell Sorting Facility, sponsored by the Louis B. Mayer Foundation, the National Science Foundation, and a Cancer Center Core Grant from the National Institutes of Health, is now fully operational. The facility consists of an Ortho Cytofluorograph 50H cell sorter (Ortho Diagnostic Systems, Inc., Raritan, N.J.) with both argon and krypton ion variable wavelength lasers. The flow cytometer is interfaced with a Data General "Nova Series" computer and a full graphics terminal with hard copy printout. This system can analyze four independent parameters simultaneously in individual cells: up to three colors of fluorescence excited by one or two wavelengths of light, and both forward and 90° light scatter to measure cell size and granularity. The optics are sensitive enough to allow cells with as few as $2 \times 10^3$ fluorescent molecules to be detected when the most common fluorochromes are used. Measurements are made at rates of $10^4$–$10^5$ cells/minute, allowing for statistically valid analyses of cells with "rare" phenotypes within minutes. Furthermore, by computer-controlled electronic sorting, one can recover relatively rare populations of living cells and subsequently culture these cells to study their functional attributes.

The year has been spent optimizing parameters and developing protocols for a variety of important biological applications. These include analysis of cell cycle compartments by determination of DNA and RNA content, and of course the detection and quantification of cell-surface molecules. We have used fluorochrome-conjugated antibodies against specific cell-surface constituents to monitor both the expression of specific gene products in transfected cells and the phenotypes of different populations of cells in development. In future years, we plan to be able to determine the fraction of cells in a population that undergo a physiological response by staining with calcium- or pH-sensitive vital dyes.

In the past year, the Cell Sorting Facility has been used by this Division to study topics as diverse as receptor biology, cell proliferation, molecular genetics, and cellular immunology and development. The potential of the instrumentation is only beginning to be tapped.

Monoclonal Antibody Facility

**Supervisor:** Paul H. Patterson  
**Staff:** To Ha Thai

The Monoclonal Antibody Facility was set up in November 1984 to provide tissue culture services for the Division of Biology. We collaborate with investigators to produce monoclonal antibodies (MAbs) for use in their experiments. We have successfully produced MAbs that specifically identify the middle temporal visual area of monkey visual cortex (John Allman's lab), antigens specific for *Drosophila* photoreceptor cells (Seymour Benzer's lab), and various epitopes on yeast tRNA ligase (John Abelson's lab). At present we are attempting to generate MAbs that recognize the upper lip representation in rat cerebellum (Jim Bower's lab), the eye discs of *Drosophila* larvae (Seymour Benzer's lab), the tRNA splicing endonuclease from yeast (John Abelson's lab), and subsets of neurons in the cat caudate nucleus (Nathan Buchwald's [UCLA] and Paul Patterson's labs). In addition to using standard hybridoma techniques, we also experiment with new methods, for example, the cyclophosphamide suppression method and electrofusion techniques.

Microchemical Facility

**Supervisor:** Suzanna J. Horvath  
**Staff:** Christine A. Hill, William D. Nelson, Ron H. Sweet, Marilyn G. Tomich

Caltech has a Microchemical Facility containing two DNA synthesizers and one peptide synthesizer. This facility synthesizes DNA and peptide fragments for members of the Caltech community. Each of the DNA synthesizers has three columns that can run independent DNA synthetic reactions with a cycle time of approximately 15 minutes. This laboratory also has equipment for the cleavage of peptide fragments from their synthetic resins. Over the past year, this facility has synthesized hundreds of oligonucleotides and 30–40 peptides for the Caltech community. In the future, we plan to expand this facility to include protein sequence analysis.
GRADUATES

FINANCIAL SUPPORT
GRADUATES

Twenty-three students in Biology were awarded the B.S. or Ph.D. degree in June 1985. Names, degrees conferred and titles of doctoral theses are as follows:

Bachelor of Science

Kurt Kendall Andersen, B.S.
John Anthony Butman, B.S. with honor
Susanna Mink Chan, B.S. with honor
James Dunn, B.S. with honor
Catherine Kei Ifune, B.S. with honor
Steven James Loyola, B.S.
Michelle Anne Mahowald, B.S.

Richard Thomas Premont, B.S.
Charles Calvin Reel, B.S. with honor
Cathy Bess Shapiro, B.S.
John Brickman Wall, B.S.
David P. Watkins, B.S. with honor
Jeanne Marie Weaver, B.S.
Christopher Yo, B.S. with honor

Doctor of Philosophy

Stuart Kilsu Kim, Ph.D. Antibody Genes, Oncogenes and Antisense Genes.

David E. Levy, Ph.D. Expression of Endogenous Retroviruses in Inbred Mice: Coordinate Regulation and Structure of Multiple Transcription Units.

Richard Sheridan Lewis, Ph.D. The Ionic Basis of Frequency Selectivity in Hair Cells of the Bullfrog's Sacculus.

Donna Lucy Livant, Ph.D. The Size of a Murine Heavy Chain Variable Region Gene Family: Implications for the Magnitude and Evolution of the VH Locus in Mouse.


Jeffrey Edward Segall, Ph.D. Chemotaxis of Escherichia coli Studied Using Ionophoretic Stimulation.

Beverly Taylor Sher, Ph.D. Studies of Class I Genes in the Major Histocompatibility Complex of the BALB/c Mouse.
FINANCIAL SUPPORT

The financial support available for the work of the Division of Biology comes from many sources: from general Institute endowment and from special endowment funds for broad areas of work; from grants or contracts with individuals, companies, foundations, and U.S. governmental agencies for specific projects; from unrestricted annual gifts; from fellowships for the support of individual scholars; and from contributions to general funds provided by Industrial Associates and Institute Associates, as follows:

Abbott Labs
*Microchemical research*

Rita Allen Foundation
*Molecular genetics research*

American Cancer Society
*Cancer research*

American Heart Association
*Cardiac research*

Anonymous Gift Fund
*Research in educational programs*

Applied Molecular Genetics, Inc. (AMGen)
*Cancer research*

The Donald E. Baxter Foundation
*Development of biological instruments*

Baylor College of Medicine
*Study of reproductive biology*

Beckman Instruments, Inc.
*Funds for equipment; Research in developmental biology*

Bing Endowment Fund, Inc.
*Professorship in behavioral biology*

Biology Research in Neurosciences
*Neuroscience research*

Biomedical Research Support (NIH)
*Biomedical research*

The James G. Boswell Foundation
*Professorship in biology*

Ethel Wilson Bowles and Robert Bowles
*Professorship in chemical biology*

Chandis Securities Co.
*Equipment, Braun Laboratories*

Norman Chandler
*Professorship in cell biology and chemistry*

Louisa Jane Church Fund
*Research in biology*

Norman W. Church Foundation
*Research in chemical biology*

The Commonwealth Fund
*General support*

Charles B. Corser Fund
*Research in chemical biology*

Roberta Crutcher
*Research in biology*

Dreyfus Foundation, Teacher-Scholar
*Biochemistry research*

Mrs. Mary Bruce Delbrück
*Research in biology*

Josephine V. Dumke Fund
*Cancer research*

Fairchild Foundation
*Fairchild scholars program*

John Douglas French Foundation
*Alzheimer's disease research*

E. S. Gosney Fund
*Research in genetics*

Frank P. Hixon Fund
*Neurobiology, physiological psychology, and related research*

Irma Hoenfly Fund
*Cancer research*

The W. M. Keck Foundation
*Neurobiology equipment*

Kraft, Inc.
*Research in biocatalysis*

Richard M. Lucas Cancer Foundation
*Cancer research*

March of Dimes
*Research in genetics*

Louis B. Mayer Foundation
*Medical research program*

The McKnight Foundation
*Neuroscience research*

Meloy Laboratories, Inc.
*Cancer research*

Monsanto Company
*Instrumentation development*

Thomas Hunt Morgan
*Professorship in genetics*

Francis L. Moseley Fund
*Cancer research*
National Institutes of Health, USPHS

Studies in basic experimental biology, animal physiology, biochemistry, biological systems analysis, biophysics, developmental biology, genetics, neurobiology, plant and cell biology, psychobiology, and virology; graduate research training, biomedical sciences support grant

National Multiple Sclerosis Society

Multiple sclerosis research

National Science Foundation

Studies in animal physiology, biochemistry, biophysics, developmental biology, genetics, neurobiology, plant and cell biology; undergraduate research participation program; Presidential Young Investigator Award

Office of the Naval Research

Visual cortex research

Pew Memorial Trust

Neuroscience research program

Gustavus and Louise Pfeiffer Research Foundation

Neuroscience research program

The President's Fund

Research and development at JPL

President's Venture Fund

Medical science

The Rockefeller Foundation

Chemical biology research

Albert Billings Ruddock Fund

Professorship in biology

Damon Runyon-Walter Winchell Cancer Fund

Cancer research

Alexander Schaeffer Fund

Research in chemistry-biology

Edwin H. Schneider Fund

Study and research in genetics

Searle Scholars Program

Molecular genetics

The Seaver Institute

Cancer research

Norton Simon

Cancer Center

Alfred P. Sloan Foundation

Neuroscience research

Alfred P. Sloan Fund for Basic Research

General support

Richard Steele

Grace C. Steele professorship of immunology

Mrs. Jean Stein

Research in psychobiology

The Stone Foundation

Research in psychobiology

A. H. Sturtevant Memorial Fund

General support

Sundry Donors

Cancer research

Systems Development Foundation

Research in neurobiology

T Cell Sciences, Inc.

Immunology research

Walter and Sylvia Treadway Fund

General support

Triton Biosciences, Inc.

Cancer research

Albert Tyler Memorial Fund

Annual lectureship in biological research

Unallocated Gifts

General support

The Upjohn Co.

Advanced biological instrumentation

Joseph G. Venable Fund

Arthritis research

Martin Webster Fund

Immunology and virology basic to the problems of multiple sclerosis

Jean Weigle Memorial Fund

General support

The Weingart Fund

Gene isolation; General support

Whitehall Foundation

Neurobiology

Cornelius Wiersma

Visiting professorship in neurobiology

Robert E. and May R. Wright Foundation

Medical science

The Zanetti Grant

Biochemistry, cancer research
Fund—Fellowship Support

Alberta Heritage Foundation for Medical Research
American Cancer Society
American Heart Association
Earle C. Anthony Fellowship
Arthritis Foundation
Myron A. Bantrell Fellowship for Scientific and Engineering Research
The James G. Boswell Foundation
British Heart Foundation
California Foundation for Biochemical Research
Cancer Research Institute, Inc.
Carnegie Institution of Washington
Centre National de la Recherche Scientifique, France
Lucy Mason Clark Fund
The Jane Coffin Childs Memorial Fund for Medical Research
Deutsche Forschungsgemeinschaft
Dysautonomia Foundation, Inc.
The Camille and Henry Dreyfus Foundation, Inc.
European Molecular Biology Organization
Fairchild Scholars Program
Federal College Work-Study Program
John E. Fogarty International Research Fellowship Program for Advanced Study in the Health Sciences (NIH)
The Anna Fuller Fund
E. S. Gosney Fund
Lawrence A. Hanson Foundation
Heisenberg Fellowship
International Union Against Cancer
Italian Government Postdoctoral
Leukemia Society of America
Life Sciences Research Foundation
MacKenzie Foundation
The Helen G. and Arthur McCallum Fund
C. J. Martin, NH and MRC Overseas Training from Australia
Muscular Dystrophy Associations of America, Inc.
National Institutes of Health, USPHS
National Multiple Sclerosis Society
National Research Council
National Science Foundation
Ann Peppers Foundation
The Procter and Gamble Co.
Gordon Ross Medical Foundation
Damon Runyon-Walter Winchell Cancer Fund
Science Research Council, England
Evelyn Sharp Fellowship
Swedish National Science Research Council
Walter and Sylvia Treadway Fund
Vern Underwood Undergraduate Scholarship
Veterans Administration
The Del E. Webb Foundation
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