Fluorescence photograph of a frozen section of the head of Drosophila melanogaster. The transverse cut goes through the eyes, optic lobes, and brain. The section is stained with two different monoclonal antibodies. One, coupled with fluorescein, shows the synaptic areas (neuropil) in green. The other, specific for nuclei, is coupled to rhodamine and shows, in red, the cellular cortex surrounding the synaptic areas. These two antibodies also stain neuropil and nuclei in the human brain (see the Abstracts by S. Benzer's group beginning on page 159).

Four-winged fly (Diptera) produced by combining three mutants of the bithorax gene complex in Drosophila. The haltere-bearing or third thoracic segment closely resembles the wing-bearing or second thoracic segment as the result of reduction in function of three genes of the complex: abx, bx$^3$, and pbx (see the Abstracts by E. B. Lewis' group beginning on page 167).
A REPORT FOR THE YEAR 1982-83
ON THE RESEARCH AND OTHER ACTIVITIES
OF THE
DIVISION OF BIOLOGY
AT THE
CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA
STAFF OF BIOLOGY 1983

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Thanks also to Nancy M. Gill, Lady Kempees, Isabella M. Lubomirski, and Kathleen F. O’Loughlin.

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 1982-June 1983, are listed separately at the end of the research reports of each group.
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Completion of the new Braun Laboratories in Memory of Carl F and Winifred H Braun was marked by formal dedication ceremonies on December 13, 1982. The photographs on the opposite page show Biology Division Chairman Lee Hood speaking at the dedication ceremony and the specially designed double-helix model given to the Braun family at the luncheon following the ceremony.

In addition to the research group of Professor Hood, which moved into Braun at the end of 1982, the Biology half of Braun is now populated by the research groups of our two new Professors, John Abelson and Melvin Simon. They will be joined in the fall of 1983 by a newly appointed Assistant Professor, Scott Emr. The other half of the building is now occupied by faculty of the Division of Chemistry and Chemical Engineering: Professors Michael Raftery and John Richards, and Assistant Professor Carl Parker.

Another significant change in the administration of the Division occurred at the beginning of the 1982-83 academic year, when Associate Professor David Van Essen succeeded Professor Jim Hudspeth as Executive Officer, with special responsibility for the Neurobiology program in the Beckman Laboratories. We are grateful to Jim for his service in this position.

HELLO--

John Abelson received his undergraduate training in Physics and then went to Johns Hopkins University, where he received his Ph.D. in Biophysics in 1965. He has moved here from the University of California at San Diego, where he was Professor of Chemistry, to join our group of molecular biologists seeking to understand the intimate mechanisms of the regulation of gene expression. John's work has primarily focused on problems associated with transfer RNA synthesis in yeast--the control of the transcription of the tRNA genes and the processing of their transcripts.

Mel Simon received his undergraduate degree in Chemistry, and then obtained a Ph.D. in Biochemistry from Brandeis University in 1963. He also has moved here from UC San Diego, where he was Professor of Biology. Mel is well known for his work on the molecular genetics of bacterial locomotion, chemotaxis, and phase variation. Mel considers this work to be part of a larger program of investigation of mechanisms for interaction between a cell's environment and its internal biochemical processes, and this program is now expanding into work on mammalian cell-surface receptors and on molecules involved in adaptation to visual stimuli.
Retiring from our faculty this year was Professor Ray Owen, after 37 years in the Division of Biology. Ray's career here has had many facets, beginning with his research in immunogenetics, which began the establishment here of immunology as a major feature of our Division's research programs. He was the Division's third long-term chairman, following Thomas Hunt Morgan and George Beadle, and served with distinction and his customary empathy in that capacity from 1961 to 1968. He served on many national committees, including the President's Cancer Advisory Panel from 1973 to 1976. He was seriously involved in many Institute responsibilities, including the committee that revolutionized the Freshman year in the early 1970s and served as the Institute's first Vice President for Student Affairs from 1975 to 1980. However, most of us will probably feel that none of these things compare in significance to the impact that Ray has had on many generations of students, as teacher, advisor, friend, and in many cases, ultimately as a colleague—an impact that derived from Ray's unique charisma and sincere interest in interacting with his students.

Ray's friends in the Division gathered together on the morning of June 20, 1983 for a party in honor of his retirement, and to express their good wishes to Ray and his wife, June. Some photographs taken at that event are shown on the opposite page. Ray confirmed that his door would still be open, and the coffee hot, in 07 Kerckhoff (after his annual month's vacation at the beach). We suspect that retirement will simply allow Ray to concentrate on doing what he enjoys most and does best—meeting individually with students and colleagues, to offer the help and support of his warmth, sincerity, scientific experience, and understanding.

Two of our faculty resigned during 1983. Associate Professor Jeremy Brockes returned to England to a position at King's College, London, and Professor James Hudspeth moved to a position as Professor at the University of California Medical School at San Francisco.

and CONGRATULATIONS---

Professor Seymour Benzer received the National Medal of Science in ceremonies at the White House on May 24, 1983. Professor Benzer is shown in the photo below receiving his award from President Reagan.

Professor Edward Lewis received the Thomas Hunt Morgan Medal of the Genetics Society of America, in recognition of his lifetime contributions to genetics.

Professor Roger Sperry received the California State Psychological Association's Plaque in recognition of distinguished scientific achievements in Psychology.

Assistant Professor Barbara Wold received a Rita Allen Foundation Scholar Award and a Searle Scholars Award.

Professor Mark Konishi gave the Eighth Clinton N. Woolsey Lecture in Neuroscience at the University of Wisconsin.

Our Division Chairman, Professor Leroy Hood was the Dreyfus Distinguished Lecturer at Indiana University, the Harvey Lecturer at The Rockefeller University, the Linus Pauling Lecturer at Stanford University, the Jesup Lecturer at Columbia University and the Syme Lecturer at the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.
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<td>Eugene Akutagawa</td>
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<td>Maria Alonso</td>
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Summary: Our laboratory has been interested for some time in the mechanisms of regulation of gene expression. We have studied these mechanisms at two levels: at the level of transcription and at the level of processing of the RNA transcript. The biological systems we have employed have been E. coli and the bakers yeast S. cerevisiae. Much of our work has been concerned with the problem of tRNA synthesis.

In yeast there are approximately 350 tRNA genes and about one-fifth of these contain an intron, a sequence of DNA which interrupts the continuity of the gene and which is not present in the mature product. We have been studying how this intron is removed in the expression of the gene. It is accomplished by an RNA processing reaction called splicing. The gene (including the intron) is transcribed to give an RNA precursor. The ends of the precursor are matured by nucleases and by the enzyme nucleotidyl transferase which adds the final three residues, C, A and A. Then the splicing is accomplished by two enzymes—an endonuclease which clips out the intron sequence and a ligase which seals the ends together. We have purified these enzymes and are studying their mechanisms of action. Ultimately we hope to isolate the genes which encode these enzymes in order to study the genetics of RNA splicing.

We also have been concerned with the transcription of tRNA genes in yeast. tRNA genes are transcribed by the enzyme RNA polymerase III. It is now clear from numerous studies in a wide variety of eukaryotic organisms that PolIII transcription is controlled by sequences in the interior of the structural gene. We have used the powerful new techniques of oligonucleotide mutagenesis to create changes in the tRNA gene which affect transcription. We have also used oligonucleotide mutagenesis to remove the intron from a tRNA gene. While removing the intron does not affect transcription, it does affect, in this case, the modification of the tRNA. A pseudouridine modification in the anticodon of tRNA1^tyt requires the intron. So in this case the intron is necessary for correct expression of the gene.

This raises a final concern of ours: What is the origin of introns in genes and what is their function? These are questions that we will be attempting to answer in the future.

PUBLICATIONS

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Support: The work described in the following research reports has been supported by:
Cenci Bolognetti Foundation, Rome
Centre National de la Recherche Scientifique
Fairchild Foundation
National Institutes of Health, USPHS

Summary: In the past year, the main thrust of our work on the human mitochondrial genetic system has been in three directions, i.e., the elucidation of the mechanisms responsible for the differential expression of the rRNA and structural genes, the development of in vitro systems for the study of mitochondrial DNA (mtDNA) transcription and mitochondrial RNA processing, and the identification of the polypeptides encoded in the unassigned reading frames (URFs) of human mtDNA. Substantial progress has been made in these three areas.

A detailed mapping and kinetic analysis of the oligo(dT)-cellulose bound and unbound transcripts of the rDNA region of human mtDNA has allowed a functional assignment for the two transcription initiation sites that had been previously identified on the heavy strand, one located very near to the 5' end of the 12S rRNA gene, and the other about 25 nucleotides upstream of the tRNA^Phe gene that flanks the 12S rRNA gene on the 5' side. Thus, the latter initiation site has been correlated with a transcription event restricted to the rDNA region, therefore terminating at the 3' end of the 16S rRNA gene, and which is responsible for the synthesis of the bulk of rRNA. The downstream initiation site has been correlated with a transcription event that instead proceeds beyond the 3' end of the 16S rRNA gene, and results in the synthesis of a polycistronic molecule corresponding to almost the entire heavy strand; this is destined to be processed to yield the mRNAs and most of the tRNAs encoded in the heavy strand. Regulation of the rate of initiation of the two overlapping transcription units is probably the main factor controlling the formation of mature rRNAs and mRNAs in human mitochondria. The most intriguing aspect of this unusual organization is that, apart from the control at the level of initiation, the fate of each transcript is determined by having started at a specific site. The investigation of the effects of intercalating drugs on mtDNA transcription in isolated organelles has provided strong support for an independent control of rRNA and mRNA synthesis in mammalian mitochondria. Further support for the model described above has come from an analysis of the steady state levels of the mitochondrial tRNAs.

In another line of investigation, mtDNA heavy and light strand transcription has been achieved in a soluble submitochondrial system using exogenous templates. An RNase P-like processing activity, capable of cleaving the E. coli tRNA^Tyr precursor precisely at the 5' end of the tRNA, has been detected in a submitochondrial fraction and shown to be associated with a large complex (in collaboration with Dr. Sidney Altman of Yale University). These in vitro systems will prove to be invaluable for the dissection of the mechanisms and regulation of transcription and RNA processing in mitochondria.

The successful application of an approach based on the use of antibodies prepared against synthetic peptides corresponding to appropriate regions of the reading frames, which is being carried out in collaboration with Dr. Russell Doolittle of the University of California at San Diego, has allowed the identification of the polypeptides encoded in three of the URFs and in the gene coding for ATPase subunit 6 in human mtDNA. Besides providing direct evidence that three of the URFs, and probably all, are expressed in mammalian mitochondria, the availability of specific antibodies will prove to be of great value for the isolation and characterization of these gene products.

Another area of investigation that is being actively pursued in our laboratory concerns the analysis of the structure and expression of the
human dihydrofolate reductase (DHFR) gene. Thus, the genomic organization of this gene has been completely elucidated. Furthermore, the occurrence in the human genome of a previously unrecognized DHFR pseudogene has been detected and its structure determined. In another line of investigation, the analysis of DHFR-specific sequences in the DNA from mouse-human cell hybrids has permitted us, in collaboration with Drs. Peter Barker and Frank Ruddle of Yale University, to make the chromosomal assignment of the human DHFR gene and the tentative assignment of three pseudogenes.

1. TWO OVERLAPPING TRANSCRIPTION EVENTS IN THE MITOCHONDRIAL rDNA REGION

Investigators: Julio Montoya, George L. Gaines

The transcription of the rDNA region of HeLa cell mitochondrial DNA has been investigated by a detailed analysis of the mapping and kinetic properties of oligo(dT)-cellulose bound and unbound transcripts synthesized in vivo and in isolated mitochondria. The data obtained have been correlated with the previous evidence pointing to the existence of two initiation sites for heavy strand transcription upstream of the rDNA region. The present and previous results have been interpreted to indicate that two distinct transcription events take place in the rDNA region. Of these, one would start ~25 bp upstream of the tRNA^Phe^ gene and result in termination at or near the 3' end of the 16S rRNA gene, being responsible for the synthesis of the bulk of rRNA. Two large oligo(dT)-cellulose unbound RNA species spanning the rDNA region, u4a and u4, are respectively a putative primary transcript of this pathway and an intermediate lacking the tRNA^Phe^ and the 5' leader. The other transcription event would start near the 5' end of the 12S rRNA gene, proceed beyond the 3' end of the 16S rRNA gene and result in the synthesis of a polycistronic molecule corresponding to almost the entire H strand. The large oligo(dT)-cellulose bound RNA b4, spanning the rDNA region, all the mRNAs, and most of the tRNAs encoded in the heavy strand, would derive from processing of this polycistronic transcript. The existence of two overlapping transcription units with distinct promoters is probably the main mechanism whereby the rates of synthesis of the rRNAs and heavy strand coded mRNAs are differentially regulated.

2. ANALYSIS OF STEADY STATE LEVELS AND METABOLIC PROPERTIES OF MITOCHONDRIAL tRNAs IN HeLa CELLS

Investigator: Michael P. King

The analysis of mitochondrial DNA transcription in HeLa cells has led this laboratory to propose a model whereby the tRNAs, rRNAs and mRNAs encoded in the heavy strand are transcribed in the form of a polycistronic transcript in which the tRNA sequences are nearly always contiguous to the rRNA and mRNA sequences. These tRNAs then provide the signal for processing to the mature species. Two types of heavy strand transcription of the mitochondrial genome in the rDNA region have been identified. One produces the polycistronic molecule that encompasses nearly the entire heavy strand. The other, which occurs at a rate 20 to 60 times higher, covers only the rDNA region and is responsible for the synthesis of the bulk of the rRNA. Three tRNA genes flank the rRNA genes—the tRNA^Phe^ gene is immediately 5' to the 12S rRNA gene, the tRNA^Val^ gene is between the rRNAs and the tRNA^Leu^ gene is immediately 3' to the 16S rRNA gene. Evidence has been obtained in this laboratory for the occurrence of two initiation sites of heavy strand transcription, one located very near the 5' end of the 12S rRNA gene and the other 20 to 40 nucleotides upstream of the tRNA^Phe^ gene. The steady state levels of tRNA^Phe^ and tRNA^Val^ are severalfold higher than the other heavy strand encoded tRNAs, supporting the hypothesis that transcription of the rDNA initiates at the site upstream of the tRNA^Phe^ gene and terminates at the 3' end of the 16S rRNA gene. We are currently determining the metabolic properties of the tRNAs mentioned above by measuring their kinetics of synthesis using labeled RNA precursors. These data will be compared to those obtained for other heavy strand encoded tRNAs and will enable us to confirm the above hypothesis.

3. TRANSCRIPTION IN ISOLATED MITOCHONDRIA

Investigator: George L. Gaines

RNA synthesis in isolated mitochondria occurs
with characteristics very similar to those shown in vivo. An investigation of this transcription system has allowed a detailed analysis of the control of ribosomal and messenger RNA production. Data gathered in part using "in organelle" synthesis have led us to postulate the idea of two overlapping promoters controlling mitochondrial transcription. This "in organelle" system will allow direct investigations of the interactions of oxidative phosphorylation, electron transport and mitochondrial gene expression.

4. IN VITRO TRANSCRIPTION IN A MITOCHONDRIAL LYSATE

Investigator: David J. Shuey

Although the pattern of mitochondrial transcription is becoming increasingly clear, the molecular details of this process remain obscure. This disparity has compelled us to develop a soluble mitochondrial in vitro transcription system as the first step towards a biochemical description of the components involved.

Accurate initiation and efficient elongation, as judged by radioactive ribonucleotide triphosphate incorporation, have been obtained in a mitochondrial lysate utilizing cloned mtDNA templates, rNTPs, and various other factors. Run-off transcription assays, with a series of truncated templates, coupled with hybridization data demonstrate that promoters for light and heavy strand transcription units are functional in this system. Salt, pH, and divalent cation requirements all clearly distinguish this activity from nuclear polymerases.

Currently my efforts are focused on the independent variation of each reaction parameter in order to maximize transcriptional activity. This analysis should also reveal any functional distinctions between the characteristics of heavy and light strand transcription.

Future studies will include the further fractionation and eventual purification and characterization of the mitochondrial polymerase(s). In addition, analysis of promoter structure, through deletion mapping and in vitro mutagenesis, is now possible.

5. IDENTIFYING THE MITOCHONDRIAL RNA PROCESSING ENZYME(S)

Investigator: Claus-Jens W. Doersen

The sequence analysis of human mitochondrial RNAs has shown, by comparison with the DNA sequence (Anderson et al., 1981), that the sequences of the heavy strand of mitochondrial DNA coding for the rRNAs and poly(A)-containing RNAs are in nearly every case immediately contiguous at both ends to a lRNA coding sequence (Crews and Attardi, 1980; Montoya et al., 1981; Ojala et al., 1981). This unique genetic arrangement, together with other data from this laboratory, supports a model whereby the heavy strand of DNA is transcribed as a single polycistrionic RNA which is processed by precise endonucleolytic cleavages, punctuated by the tRNA sequences, to yield the mature rRNAs, poly(A)-containing RNAs, and tRNAs.

Using an E. coli tRNA\textsuperscript{TYT} precursor (in collaboration with Dr. S. Altman\textsuperscript{*}) an endonucleolytic activity similar to E. coli RNase P (Altman et al., 1980) has been identified in fractionated mitochondrial preparations. This activity makes a precise endonucleolytic cleavage at the 5' end of the tRNA sequence. Experiments utilizing an in vitro synthesized RNA containing mitochondrial tRNA sequences (Biology 1982, No. 3) are in progress to test if other mitochondrial RNA processing activities are associated with the RNase P-like activity.

References:

\textsuperscript{*}Department of Biology, Yale University, New Haven, Connecticut.
6. THE UNIDENTIFIED READING FRAME A6L IS EXPRESSED IN HUMAN MITOCHONDRIA

Investigators: Paolo Mariottini, Anne Chamyn, Giuseppe Attardi, Dennis Trovato*, Donna D. Strong*, Russell F. Doolittle*

Antibodies prepared against chemically synthesized peptides predicted from the DNA sequence have been used to detect human mitochondrial gene products. In particular, antibodies directed against either the NH₂-terminal decapeptide or the COOH-terminal undecapeptide of cytochrome c oxidase subunit II (COII) were both very effective in immunoprecipitating the previously identified COII polypeptide from an SDS lysate of mitochondria from HeLa cells. Similarly, antibodies directed against the COOH-terminal nonapeptide of the putative polypeptide encoded in the unidentified reading frame A6L, which overlaps the ATPase 6 gene, immunoprecipitated specifically a component (#25) of the HeLa cell mitochondrial translation products; antibodies directed against the NH₂-terminal octapeptide also precipitated protein 25, although less efficiently. The size of protein 25, as estimated from its electrophoretic mobility, is compatible with its being the URFAG6L product. Furthermore, a fingerprinting analysis of this protein after trypsin digestion has given results consistent with this identification.

*Department of Chemistry, University of California at San Diego.

8. PRESENCE OF MITOCHONDRIAL DNA SEQUENCES IN THE HUMAN NUCLEAR GENOME

Investigators: Barry J. Maurer, Michael King

Recently the nuclear genomes of several organisms, including sea urchin and locust, have been shown to contain DNA sequences of mitochondrial origin. The presence of mitochondrial sequences in the human nuclear genome has been investigated by Southern blot analysis using human mitochondrial probes cloned in the single-stranded DNA phage M13. Results indicate that DNA sequences from several mitochondrial regions exist in the human nuclear genome.

9. DHFR GENE ORGANIZATION IN A METHOTREXATE-RESISTANT HUMAN CELL LINE

Investigators: Jiyoung K. Yang, Jeffrey N. Masters

We previously reported (Biology 1982, No. 10) isolating the complete dihydrofolate reductase (DHFR) gene from Charon 4A libraries of the methotrexate-resistant variant human cell line, VA₂B-6A3. However, further analysis showed that one of the clones containing the middle portion of the gene was unstable and underwent rearrangement rapidly. We constructed a λ31 library using an MboI partial digest of VA₂B-6A3 chromosomal DNA, and screened the library with ³²P-labeled probes containing previously isolated 5' end or middle segments of the DHFR gene, ³²P-labeled by nick translation products, the reaction being inhibited by the specific peptide. Similarly, antibodies directed against the COOH-terminal undecapeptide of the putative URF1 product or against the COOH-terminal heptapeptide of the presumptive URF3 product were effective in immunoprecipitating specifically component 12 or, respectively, component 24 of the mitochondrial translation products. The sizes of proteins 17, 12 and 24, as estimated from their electrophoretic mobilities, are compatible with their being the products of the ATPase 6 gene, URF1 and URF3, respectively.

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translation. Several λ clones containing the missing middle portion of the gene were isolated, and one of these clones fortunately was stable. In addition, clones containing 5' flanking sequences as well as 3' portions of the gene were isolated from this library. Altogether the isolated cloned segments of the entire DHFR gene and its flanking sequences encompass about 50 kbp. The size of the gene itself is about 30 kbp. One of the exons has been sequenced and the others are being sequenced at the present time.

Subcloned fragments of various portions of the DHFR gene were utilized for restriction mapping and for S1 analysis of exons. S1 analysis of the 5' end fragment containing the first exon showed that two fragments, 132 bp and 105 bp, were protected by cytoplasmic poly(A)+ RNA from VA2-B6A3 cells. The relationship of this observation to the existence of multiple DHFR mRNA species (Morandi et al., 1982) is being investigated.

References:

10. A HUMAN DIHYDROFOLATE REDUCTASE PSEUDOGENE AND ITS RELATIONSHIP TO THE MULTIPLE FORMS OF SPECIFIC MESSENGER RNA

Investigators: Jeffrey N. Masters, Jiyoung K. Yang

The presence of dihydrofolate reductase (DHFR) specific sequences which, in contrast to the normal DHFR gene, are not amplified in a methotrexate-resistant cell line, has been detected in the DNA from human sperm and from several human cell lines. DNA fragments containing some of these sequences have been isolated from a cosmid library of human sperm DNA. One of these fragments contains a DHFR pseudogene (HD1) which completely lacks introns, has 92% sequence homology to the corresponding region of normal DHFR cDNA, but exhibits several alterations which make it non-functional.

The sequence analysis of the inserts of four different plasmids containing the reading frame and varying lengths of the 3' noncoding regions of human DHFR-specific cDNA has revealed that the 3' noncoding segments are all collinear in their corresponding portions. Furthermore, the data have indicated that the cDNA of one of the plasmids has probably derived from the smallest of the three main human DHFR mRNAs, the 0.8 kb mRNA, the cDNA of two others, from the 1.0 kb mRNA, and the cDNA of the fourth, from a longer mRNA. These results are consistent with the idea that the multiple forms of DHFR mRNA in human cells derive from the same gene by different transcription or RNA processing events. Moreover, the sequence comparison between the HD1 and the different DHFR cDNAs clearly indicates that, if an mRNA intermediate has participated in the formation of this pseudogene, a form of mRNA larger than the 1.0 kb mRNA, probably the 3.8 kb mRNA, must have been involved.

11. CHROMOSOMAL ASSIGNMENT OF THE HUMAN DIHYDROFOLATE REDUCTASE GENE AND PSEUDOGENES

Investigator: Barry J. Maurer

Recently, several cell variants resistant to high concentrations of methotrexate (MTX) have been isolated from two different human cell lines, Hela BU25 and VA2-B. As described for other mammalian cultured systems, these cell lines hyperproduce dihydrofolate reductase (DHFR), the target enzyme of MTX as a result of selective amplification of the DHFR structural gene. One of the most striking features of these MTX-resistant human variants is the pleomorphism of chromosomal alterations that they exhibit. These include the presence in all the variants of a highly variable number of double minute chromosomes (DMs), the occurrence in some variants of a homogeneously staining region (HSR) containing DHFR genes in one or more identifiable chromosomes, in some cases concurrent with the presence of double minute chromosomes (see Abstract No. 12) and the occurrence in some variants of a duplicated set of chromosomes in most cells of the population. In order to understand the nature and mechanism of formation of these chromosomal anomalies, it is important to establish the chromosomal location of the unamplified human DHFR gene. Such a chromosomal assignment is also of interest in the general context of human gene mapping. The recent cloning and characterization of human DHFR cDNAs and the identification in EcoRI digests of human genomic DNAs of the fragments containing the exons of the amplifiable DHFR gene has made it possible to investigate the chromosomal
location of the DHFR gene by DNA transfer hybridization analysis of genomic DNA from human-rodent cell hybrids segregating human chromosomes, and by in situ hybridization analysis of metaphase chromosome spreads. In collaboration with Drs. Peter Barker and Frank Ruddle (Yale University), analysis of hybrids has allowed the unambiguous assignment of the human DHFR gene to chromosome 5 and the tentative chromosomal assignment of several of the other variant gene fragments.

12. LOSS OF DOUBLE MINUTE CHROMOSOMES AND APPEARANCE OF HOMOGENEOUSLY STAINING REGIONS IN A HUMAN MTX<sup>R</sup> CELL LINE, VA<sub>2</sub>B-6A3

Investigator: Barry J. Maurer

A karyotype analysis of several methotrexate-resistant (MTX<sup>R</sup>) human cell variants isolated in this laboratory from VA<sub>2</sub>-B cell lines (Masters et al., 1982) has revealed the presence of double minute chromosomes (DMs) which have been shown to contain at least some of the amplified genes for dihydrofolate reductase (DHFR) by in situ hybridization (Biology 1981, No. 12). One variant, VA<sub>2</sub>B-6A3, exhibits, in addition to DMs, a chromosome bearing a homogeneously staining region (HSR). Upon extended growth in methotrexate, VA<sub>2</sub>B-6A3 loses almost all of its DMs and acquires two additional HSRs in distinctive chromosomes. An extensive analysis of this phenomenon has been carried out on time points extending over a 16-month period. Karyotype analysis has been performed to study the kinetics of DM loss and HSR acquisition. Growth rate studies are being conducted to explore whether a selective advantage is conferred upon the cell by DHFR genes located on HSRs as compared to those residing in DMs. In situ hybridization experiments using a 125I-dCTP-labeled human DHFR cDNA probe have been conducted to determine the DHFR gene location at various stages during the development of MTX resistance. Results indicate that the acquisition of the two later appearing HSRs is sequential and "catastrophic," with no developmental intermediaries.

References:

13. A NEW TYPE OF EcoRI POLYMORPHISM OF THE HUMAN RIBOSOMAL DNA

Investigators: Monica Muttes*, Susan Shu-Ai Lai, Julio Montoya

Several clones of rDNA have been isolated from an adult human liver DNA A Charon 4A library by using cDNA probes synthesized from human 18S and 28S rRNA. The insert of one recombinant Charon 4A clone contained, besides the already known 5.7 kb EcoRI fragment of rDNA comprising the major portion of the 18S rRNA gene and all the external transcribed spacer, a previously unidentified EcoRI fragment of rDNA of 8.5 kb in size. DNA transfer hybridization experiments utilizing EcoRI digests of the human DNA used to construct the library and of another human DNA showed that the cloned 8.5 kb fragment corresponds in the rDNA to a segment of the same size, which is adjacent to the 5' end of the 5.7 kb segment. In the majority of the rDNA repeated units of both DNAs, the 8.5 kb segment is flanked at its 3' end by an EcoRI site, previously recognized as optional; however, only a small fraction of the repeating units exhibiting this site also possess an EcoRI site at the 5' end of the 8.5 kb segment. The presence of the 8.5 kb EcoRI fragment in a minority of the rDNA repeats defines a hitherto unidentified type of EcoRI polymorphism.

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Publications


Summary: Interest in our laboratory has for several years centered on studies at the molecular level of the organization and expression of the genome in higher animals. We use sea urchin eggs and developing embryos, including larvae carried through metamorphosis to maturity as a model system in much of our work. We are utilizing recombinant DNA techniques for analysis of single genes and gene families, and have constructed libraries of genomic DNA from several sea urchin species. Thus we are able to measure the conservation of certain sequences through evolution and between sea urchin populations, as well as the distribution of single copy and repeated sequences in their vicinity in the genome. Recently, we have been successful in

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developing a method of transforming sea urchin genomes by introducing cloned DNA sequences into eggs.

Maternal RNA (RNA sequences stored in sea urchin eggs) and RNA extracted from embryos are used for study of the expression of sea urchin genes during oogenesis and embryogenesis. The role of maternal transcripts, in which gene transcripts are interspersed with transcripts of other single copy and repeated sequences, is being studied. We also are working to prepare cloned libraries in an expression vector, for the selection of specific genes using antibodies to specific proteins.

The sequence organization and expression of the sea urchin actin gene family have been investigated in great detail, and we are using a variety of approaches to obtain clones of genes coding for other specific sea urchin proteins such as the bindin protein of sperm. Other particular projects are concerned with DNA sequence organization, and gene expression, in a number of eukaryotes. In all, our aim is to better understand the mechanisms of gene control, and the relationships between DNA sequences, in higher animals.

14. INTERSPERSED MATERNAL RNA IN SEA URCHINS

Investigators: Frank J. Calzone, Howard T. Jacobs, Caleb E. Finch, Eric H. Davidson

The majority of the RNA transcripts containing message sequences in the sea urchin egg more closely resemble unprocessed or partially processed gene transcripts than they do mature mRNA (see Calzone et al., 1983 for a short review). These transcripts are interspersed with untranslatable short repetitive sequence elements (Posakony et al., 1983) and have been called "interspersed" maternal RNA to denote this fact. RNA transcripts resembling interspersed maternal RNAs are found in the nucleus, but not the cytoplasm of cells in the late embryo and adult. It is likely that interspersed maternal RNAs are a source of mRNA translated on polysomes of the early embryos. Cell free translation studies and microinjection experiments with Xenopus oocytes have demonstrated that interspersed maternal RNA isolated from Xenopus oocytes is not translatable or loaded onto polysomes (Richter et al., 1983). Thus interspersed maternal RNAs require processing if they are to be translated after fertilization. It is possible that the translational inhibition is due to short repeats which interrupt protein coding sequences, but this has not been demonstrated. A number of cDNA clones have been obtained which represent several different interspersed maternal RNAs (Posakony et al., 1983). We have used single copy portions of these cDNAs to select genomic DNA clones from a variety of λ libraries of S. purpuratus sperm DNA. We are using the genomic clones to analyze the structure of the interspersed maternal RNA represented by the cDNA clones.

References:

15. STRUCTURE AND DEVELOPMENTAL FATE OF SEA URCHIN INTERSPERSED MATERNAL RNA

Investigators: Howard T. Jacobs, Tracy T. Furutani, Frank J. Calzone, Eric H. Davidson

We are investigating the relationship of interspersed maternal RNA (see Abstract No. 14) to actual embryo messages. To this end we are studying the sets of maternal transcripts derived from individual transcription units of the sea urchin genome, which give rise to abundant embryo mRNAs for which cloned cDNAs are available. A number of such (single-copy) cDNAs that have been investigated react in RNA gel blot hybridizations with multiple transcripts in egg poly(A) RNA, including high molecular weight species which are essentially all of the interspersed class.

One transcription unit which has been studied in greater detail (TrU 154) encodes a 1.6 kb transcript which is abundant in embryo polysomal poly(A) RNA. In egg RNA, however, a major transcript of 7.5 kb is also detected, in addition to other minor species of 6.0 and 3.8 kb. The concentration of the 7.5 kb transcript, only slightly less than that of the 1.6 kb species in total egg RNA, appears to decline sharply during
the first hours of embryological development, while that of the 1.6 kb transcript shows a modest increase. RNA gel blot hybridizations have shown that some sequences represented in the 7.5 kb RNA correspond with intervening sequences which are spliced out of the 1.6 kb transcript. Nucleotide sequencing has shown that these regions contain numerous translational stop signals in all possible reading frames. The 6.0 kb and 3.8 kb species may be partially spliced intermediates. The 7.5 kb transcript is largely, if not exclusively non-polyadenylated, copurifies with repeat-containing RNA when total egg RNA is self-annealed and fractionated by ethanol-cellulose chromatography, and is colinear with genomic DNA in the region studied thus far.

Our working hypothesis is that the 7.5 kb transcript represents an interspersed RNA which is an unprocessed precursor of the 1.6 kb message, and which may be productively polyadenylated and spliced during early development, when transcription is minimal. If further experiments confirm this interpretation, this would indicate a role for at least some of the interspersed RNA stored in the egg cytoplasm--i.e., a message precursor.

16. INJECTION OF DNA SEQUENCES INTO SEA URCHIN EGGS

Investigators: Andrew P. McMahon, Barbara R. Hough-Evans, Constantin N. Flytzanis, Karen S. Katula, Edward M. Nolan, Eric H. Davidson

DNA transformation is a powerful technique for the study of gene expression. We have succeeded in developing a microinjection technique for DNA transformation of the sea urchin Strongylocentrotus purpuratus. Cloned DNA sequences are injected by means of a continuously flowing microneedle. The recipient unfertilized eggs are fixed to the bottom of a shallow dish of filtered seawater. Each egg receives a few picoliters (about 1-2% of its volume) of a solution containing some 3000 DNA molecules. The injected eggs are then fertilized, and embryos are cultured to the desired stage of development. About half the embryos survive to the pluteus stage, and 75% of these can be carried through metamorphosis. Four- to five-week-old larvae have been assayed for the presence of injected sequences; at this stage the animal contains about 50,000 cells, and a single copy of the injected sequence per genome can be detected. Eight different plasmids have been tested in the system, including Drosophila P-factor plasmids, plasmids carrying a neomycin-resistance gene, and a cloned putative sea urchin transposon. The fraction of injected embryos in which the introduced DNA can be detected after four weeks of development varies from 3% to more than 60%, depending on which cloned DNAs were injected. Experiments are in progress to characterize this sea urchin transformation system and to determine the molecular basis for retention and replication of particular DNA sequences.

17. DNA TRANSFORMATION IN THE SEA URCHIN

Investigators: Constantin N. Flytzanis, Karen S. Katula, Edward M. Nolan, Andrew P. McMahon, Barbara R. Hough-Evans, Eric H. Davidson

We have constructed and tested several plasmid vectors for transformation (through microinjection) of sea urchin eggs. All vectors contain the necessary sequences for replication and selection in E. coli cells, from the plasmid vector pBR322. All vectors also contain an assemblage of the neomycin phosphotransferase gene from the prokaryotic transposon Tn5 ("neo") and the eukaryotic promoter region and poly(A) addition site from the thymidine kinase gene of the herpes simplex virus (\(t_u\) and \(t_k\)). One set of the constructed vectors is based on the transposable element \(P\), from Drosophila melanogaster. In one vector the \(t_u\)-neo-tk assemblage was inserted between the inverted repeats of the \(P\) element [provided by C. Rubin and A. Spradling (Rubin and Spradling, 1982)]. In others, different segments from the \(P\) element were inserted in a plasmid containing the \(t_u\)-neo-tk assemblage. In another set of vectors the same \(t_u\)-neo-tk assemblage was inserted between the inverted repeats of a transposable element isolated from the sea urchin Strongylocentrotus purpuratus (a gift of D. Lieberman and L. Kedes, Stanford University).

All vectors were microinjected into eggs of S. purpuratus and the presence of the injected DNA in four-week-old larvae was detected by dot blot
Some of the analysis (see Abstract No. 16). Some of the injected larvae were allowed to metamorphose and DNA was prepared from whole baby urchins four weeks later. Genome blots were carried out using the injected plasmids as probes for the hybridization. The injected sequence was detected in 10-15% of the metamorphosed animals. In all cases where the DNA could be detected it was also integrated in the genome and no extrachromosomal plasmids were found. In some animals only a single copy of the injected sequence was integrated, and in some multiple copies were integrated at presumably different sites in the genome. We have recently constructed genomic libraries from individuals with integrated injected sequences in an effort to analyze the sites of the genome where the integration took place and the sequences in our vectors that were responsible for integration. We are currently investigating the expression of the neomycin phosphotransferase gene in the injected embryos using a modification (developed by Nevis Fregien, Caltech Division of Chemistry) of the enzyme assay used with bacterial extracts (Haas and Dowding, 1975).

References:

18. CLONING SEA URCHIN GENES OF DEVELOPMENTAL INTEREST USING THE VECTOR Agt11

Investigators: Henry M. Sucov, John J. Robinson, Boning Gao, Terry L. Thomas, Eric H. Davidson

We have begun a study of the sequence organization and expression of sea urchin genes that code for proteins of developmental interest. One such protein is a specific product of embryonic mouth-forming cells. At the transition between gastrula and pluteus stages in sea urchin development, the growing gut tube bends and comes in contact with the outer wall of the embryo. Those ectodermal cells located at the point where contact is made are induced to differentiate, eventually forming the larval mouth. Antibodies against a protein expressed in these cells following induction have been made available to us by the laboratory of David McClay (University of North Carolina). Hyalin is another protein both developmentally regulated and cell specific in its pattern of expression. This protein is synthesized in the oocyte and stored in the unfertilized egg. It is released at fertilization and coats the surface of the embryo during early development. Hyalin is not synthesized during cleavage, but synthesis begins again in ectodermal cells of the mesenchyme blastula and continues to the pluteus stage.

We are preparing cDNA libraries from poly(A)+ RNA of gastrula stage embryos, using the lambda expression vector Agt11. This vector, developed in the Ron Davis laboratory at Stanford, contains a unique EcoRI restriction site located within the lac Z gene. Complementary DNA inserted into this site in the correct reading frame gives rise to a hybrid protein that can be detected using labeled antibodies. The libraries will initially be screened with antibodies to larval mouth protein and hyalin for isolation of these genes.

19. ORGANIZATION AND EXPRESSION OF ACTIN GENES IN THE SEA URCHIN

Investigators: Samuel J. Rose, Rosemary J. Shott, James J. Lee, Marvin J. Rosenberg, Terry L. Thomas, Eric H. Davidson

We are investigating the actin gene family of the sea urchin Strongylocentrotus purpuratus as a model for the organization of multigene families with respect to their differential expression and evolution. Genomic DNA blotting experiments and characterization of lambda genomic isolates show that there are approximately eight non-allelic genes. These genes can be subdivided into four categories based upon homologies in the 3' untranslated regions of the mRNAs (Scheller et al., 1981). These 3' untranslated regions of lambda isolates have been subcloned and used as probes to investigate 1) the differential expression of genes during early development and in adult tissues, 2) the organization of these genes within the S. purpuratus genome, and 3) the evolutionary conservation of genes within and between different sea urchin genera.

RNA gel blots have shown that the majority of actin genes are expressed at some stage in the life cycle of the sea urchin. However, each gene shows a different pattern of regulation during early
development and between various adult tissues. Overlapping genomic lambda isolates shows that three of the actin genes are linked over a 24-kb region. These three genes represent two of the actin subcategories. The expression of each gene during early development and in differentiated adult tissues is regulated in a very specific manner which differs for each of the three genes. In this case, therefore, gene linkage appears to have no obvious functional role in the regulation of gene expression. The actin gene set therefore provides an excellent model for studying specific sequence requirements of developmentally regulated genes using, for example, in vitro mutagenesis/DNA transformation techniques.

Genomic blot analyses of DNAs from individuals of Strongylocentrotus droebachiensis, S. franciscanus, Tripneustes gratilla and Lytechinus pictus using coding and 3' untranslated specific probes from S. purpuratus are establishing both the evolutionary relationships between the actin subtypes and the approximate number of actin genes among these sea urchin species. Observations demonstrating both conserved and diverged sequence elements among the actin gene subfamilies are allowing a molecular description of the evolution of this very important and ubiquitous gene.

Reference:

20. "EARLY" AND "LATE" GENES

Investigators: Steven A. Johnson, Eric H. Davidson

Recent work in our laboratory has led us to propose that genes be placed in two very broad classes, based on their possible mode of regulation. Early genes are those whose transcripts are present in maternal RNA and embryonic or adult nuclear RNA, but may not be present in embryonic or adult cytoplasmic RNA. Late genes do not contribute to the maternal transcript population and are transiently regulated during development, possibly in a tissue-specific manner.

Wold et al. (1978) found that while less than 20% of blastula cytoplasmic RNA complexity is still present in any S. purpuratus adult tissue cytoplasmic RNA preparation, virtually all of the blastula message set is found in both adult tissue nuclear RNA and total egg RNA. While this work focused mainly on the rare message class, more recent work (Flytzanis et al., 1982) has ascribed similar characteristics to early gene transcripts of moderate to high prevalence where the prevalence remains relatively constant throughout embryogenesis. In contrast, Flytzanis et al. (1982) also described a number of transcripts that are not detectable in maternal RNA, but whose prevalence increases drastically at a later embryonic stage. Many of these late gene transcripts are quite specific and disappear after a certain period of development has occurred; others may be specific to an embryonic tissue or an adult cell type.

A hypothesis that follows from these findings suggests that early and late genes are regulated in fundamentally different ways. In general, an early gene is transcribed at a relatively constant level throughout oogenesis, embryogenesis, and into adulthood, and the control of cytoplasmic entry is thus proposed to be at a posttranscriptional level. A late gene is primarily regulated at the transcriptional level and may or may not be susceptible to various posttranscriptional controls.

One way to test this hypothesis involves measuring transcript prevalence of early and late genes in both nuclear and cytoplasmic RNA preparations from different embryonic stages and adult tissues. We would predict that early gene transcripts will be present in any nuclear RNA examined, but most likely not be present in advanced embryo or adult tissue cytoplasmic RNA preparations. Conversely, a late gene transcript, when present in the nuclear RNA of a certain embryonic stage/adult tissue will always be present in the cytoplasmic RNA of that embryo or adult tissue.

To this end we have engaged in the isolation of intact, blot quality RNA from nuclear and cytoplasmic preparations of various embryonic stages and adult tissues. These RNA preparations will be tested with various early and late cloned gene probes by standard Northern gel blot hybridization techniques.
References:

21. A STUDY OF THE SEA URCHIN "BINDIN" GENE
Investigators: Boning Gao, Eric H. Davidson
Bindin (the sperm-borne binding protein) is a major protein in the acrosome of sea urchin sperm. It binds to the receptor on the egg membrane and renders fertilization species-specific. The molecular weight of bindin is 30,500 daltons. The protein has been sequenced (Glabe and Vacquier, 1978; Vacquier, unpublished data). We are interested in its gene structure and regulation.
We isolated poly(A)+ RNA from sea urchin testes, translated it in an in vitro translation system, and immunoprecipitated the proteins with antibody against bindin. We have found one single 60,000-dalton protein which is immunoprecipitated by bindin antibody. Further immunocompetition experiment has strongly suggested that this protein is a bindin-related protein or bindin precursor.
We also made a testes cDNA library which contains 200,000 recombinant clones. We are currently screening this cDNA library with synthetic probes (a mixture of 17-nucleotide DNA fragments synthesized to correspond to the amino acid sequence for which we thank Suzanna Horvath in Dr. Hood's lab).

Reference:

22. A MOVEABLE ELEMENT IN A SEA URCHIN Tu88 GENE COMPLEMENT
Investigators: Steven A. Johnson, Roy J. Britten
The sea urchin Strongylocentrotus purpuratus (Sp) gene designated Tu88 codes for a maternal transcript whose cytoplasmic prevalence and post-transcriptional processing are developmentally regulated during early development. We have previously reported in these abstracts on the isolation and apparent high degree of sequence conservation of the Tu88 counterpart from S. droebachiensis (Sd). We also reported that a small insert was present in a single copy region of the Sd gene. This insert, termed SJ1, has been characterized and sequenced.

The hybridization of nick-translated dRH1.5, the Sd single copy region containing the insert SJ1, to a blot of restricted genomic DNA suggests that SJ1 is repeated a few hundred times in Sp, Sd and to a lesser extent in S. franciscanus (Sf). SJ1 is organized in an interspersed fashion, and is mobile, at least in an evolutionary sense, since it is present in Sp but not at the equivalent location initially discovered in Sd. We have sequenced the corresponding regions in Sd and Sp that contain and lack the SJ1 element, respectively. Our evidence shows that SJ1 is a mobile repeat with a length of 118 bp not including homologous terminal direct repeats of 13 bp. We are presently screening genome libraries from different Sp individuals for examples where the SJ1 element is present at a certain genomic position in one individual, but absent from that same position in another individual. This would provide direct evidence for the movement of SJ1 within the Sp species. Furthermore, such examples will strengthen the developing tenet that polymorphism may be due to moveable elements as well as point mutations in higher animals.

Reference:

23. COMPARISON OF SEA URCHIN AND HUMAN MITOCHONDRIAL DNA
Investigators: John W. Roberts, John W. Grula*, James W. Posakony**, Richard Hudspeth*, Eric H. Davidson, Roy J. Britten
Recombinant plasmids SpG30 and SpP389 were originally isolated as cDNA clones of prevalent sea urchin (S. purpuratus) embryo poly(A)+ mRNAs. The cloned sequences were subsequently identified as representing mitochondrial transcripts by blot hybridization to human mtDNA restriction fragments and by direct nucleotide sequence analysis. Plasmid SpP389 carries approximately 1200 nucleotides from the 3' end of 16S rRNA; SpG30 carries approximately 700 nucleotides from the 3' end of the cytochrome oxidase subunit I (CO I) gene.

Plasmid SpG30 was used as a hybridization probe to screen a recombinant library constructed from a partial EcoRI digest of S. franciscanus DNA. Three
full-length clones of sea urchin mtDNA were isolated and mapped and the locations of the CO I and 16S rRNA genes determined. The CO I and 16S rRNA genes are adjacent in sea urchin mtDNA. In human (Anderson et al., 1981), bovine (Anderson et al., 1982), and mouse (Bibb et al., 1981) mtDNA, the 16S rRNA and CO I genes are separated by a 2.5-kb segment containing URF-1 and URF-2.

To compare overall sequence organization, radiolabeled restriction fragments of sea urchin mtDNA were used as hybridization probes on blots of restriction digests of human mtDNA. The reactions of sea urchin DNA fragments confirm that the 16S rRNA and CO I genes are adjacent in sea urchin mtDNA, and suggest that the 16S rRNA and 12S rRNA genes are separated by a region containing URF-1 and URF-2. In mammalian mtDNA the 12S and 16S rRNA genes are adjacent. Although gene order is different in Drosophila mtDNA, the two rRNA genes are likewise adjacent (O'Clergy et al., 1982). Thus we conclude that the difference in gene order in sea urchin mtDNA reflects a rearrangement that occurred in the sea urchin lineage after sea urchins and mammals last shared a common ancestor, more than 500 million years ago.

References:
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**Department of Biochemistry and Biophysics, Harvard University.

24. INTERSPERSED MIDDLE FREQUENCY REPEATS IN HUMAN, GORILLA, AND CHIMPANZEE DNA

Investigators: John W. Roberts, Richard L. Hudspeth, Roy J. Britten

The distribution of Alu family and middle frequency repeats in the human genome has been estimated using randomly chosen genomic DNA clones from a human recombinant library. The average spacing between Alu repeats is approximately 4.3 kb, corresponding to a repetition frequency of 700,000 Alu repeats per human genome. The average spacing between middle frequency repeats of repetition frequency approximately 100-200 is about 15 kb. There are at least 170,000 middle frequency repeats in the human genome. Preliminary statistical analysis of a gorilla genomic DNA library suggests that the middle frequency repeats are similar in sequence and distribution in human and gorilla DNA. There appear to be fewer copies of Alu family repeats in the gorilla genome than in the human.

Human interspersed repetitive sequences have been isolated and cloned using methods previously employed to isolate and clone sea urchin interspersed repeats (Scheller et al., 1977). Human genomic DNA sheared to approximately 2-3 kb fragment length was denatured, renatured to Cot 250 (the Cot 1/2 for human single copy DNA is approximately 3000), digested with S1 nuclease, and the S1-resistant duplex DNA fractionated on a Sepharose CL-2B gel filtration column. Small libraries of the short (mode length less than 0.3 kb) and intermediate (mode approximately 0.6 kb) length fractions were prepared by the addition of synthetic BamHI sites followed by insertion into the BamHI site of plasmid pUC9. Representative examples of human mid-frequency repeats will be characterized with regard to repetition frequency and sequence divergence of family members in the human, chimpanzee, and gorilla genomes. Recombinant clones of genomic occurrences of these repeats will be examined for evidence of transposability.

Reference:

25. CONTROL OF GENE EXPRESSION IN ONE-CELL AND TWO-CELL MOUSE EMBRYOS

Investigators: Lajos Pikó, Kerry B. Clegg*

Several lines of evidence indicate that protein synthesis in the one-cell fertilized mouse egg is carried out mostly, if not exclusively, by
maternally inherited components. However, soon after the first cleavage division (about 24 hours after fertilization), a new pattern of protein synthesis emerges which is dependent on RNA synthesis by the embryonic genome. We have studied the RNA synthetic activity of embryos during this period of transition, taking advantage of the high specific activity of the ATP-pool when 3H-adenosine is used as a labeled precursor (Clegg and Pikó, 1982). We have found that heterogeneous nuclear RNA is synthesized at a high rate, about 0.05-0.1 pg/min/cell, in both one-cell and two-cell embryos. Only a very small fraction, about 1-2%, of this RNA is converted into stable poly(A)+ RNA in the one-cell embryo whereas 5-10% is converted in the two-cell. Therefore, the control of embryonic gene expression at this stage appears to reside at least in part in posttranscriptional events. A striking feature of the one-cell embryo is the very high rate of synthesis of poly(A) tracts, about 4×10⁶ tracts/min/embryo, due almost entirely to cytoplasmic adenylation of previously non-polyadenylated stored RNA. The data suggest a turnover of the poly(A)+ RNA population in one-cell embryos as a result of polyadenylation of new RNA sequences and degradation of some of the pre-existing poly(A)+ RNA. In the two-cell embryo, a large fraction of poly(A) synthesis is nuclear, reflecting increased synthesis of embryo-derived poly(A)+ RNA.

Reference:

26. EXPRESSION OF RETROVIRUS-RELATED SEQUENCES IN EARLY MOUSE EMBRYOS

Investigators: Lajos Pikó, Kent D. Taylor*

A characteristic feature of early mouse development is the transient appearance of large numbers of intracisternal A-type particles (a retrovirus-like structure) in the 2- to 8-cell embryo. Morphologically similar particles are also abundant in mouse plasmacytomas and other tumors. The A-particle gene, typically about 7 kb in size, has a structure similar to transposable genetic elements and is repeated 500-1000X in the mouse genome.

We have assayed for the presence of A-particle-related RNA sequences in early embryos by a dot hybridization procedure using purified 32P-labeled fragments of the A-particle gene (cloned in the single-stranded DNA phage M13). A-particle-related RNA was detected in the embryos at all stages from the one-cell to the preimplantation blastocyst (about 64 cells). The amount of A-particle-related RNA per embryo increased about 10X from the two-cell stage to the blastocyst and was estimated at about 0.5% of the total poly(A)-containing RNA at these stages. As there is no correlation between the amount of A-particle RNA and the number of morphologically detectable particles in the embryos, the particles may represent only a transition form of the expression of the A-particle genes. The role of these genes in embryo development is as yet obscure.

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The primary reason that so little is known about these molecules is that it has been difficult for anyone to isolate such scarce cell-surface proteins in amounts suitable for conventional analysis. We have approached this problem by: (1) development of highly sensitive instrumentation capable of analyzing very small quantities of proteins; (2) analysis of molecules from cultured (tumor) cell lines, an approach that provides substantial amounts of a cloned population of cells that express the specific proteins of interest; (3) using the amino acid sequence information for computer-assisted searches for evolutionarily and functionally related molecules; and (4) using synthetic DNA probes to facilitate the cloning of genes of particular interest.

In previous years we invested considerable effort in the design and construction of microchemical instrumentation. That program has been successful and the desired instruments are now in routine use. As an example, the high sensitivity of the new sequencing instrument now enables us to obtain useful amino-terminal sequence information on very small quantities of proteins isolated after
analytical scale separation by SDS-gel electrophoresis.

Several cell-surface receptors that are differentially expressed on specific cell lineages are under study in collaborative projects that are discussed in the abstracts that follow. Although not discussed in an abstract here, we have recently initiated a project dealing with cell-surface proteins that appear to play a role in specific neuronal migrations during embryogenesis. We not only wish to characterize such molecules but also the DNA sequences in and near the relevant structural genes. Our aim in each of these closely related studies is to increase our understanding of cell-surface proteins, the genes that code for them and the genetic mechanisms that program their expression in specific cell lineages.

References:

27. MICROCHEMICAL STUDIES OF THE MURINE CELL-SURFACE RECEPTORS LFA-1 AND Mac-1

Investigators: David B. Teplow, Timothy A. Springer*, William J. Dreyer

It is likely that related families of cell-surface proteins are involved in the orderly migration and association of cells during embryogenesis and development. One of the best studied systems in which large families of cell-surface proteins have been described is the immune system. Recently, a monoclonal antibody was produced which could interfere with antigen-specific T cell responses, presumably by blocking the function of a molecule involved in cell-cell recognition (Kürzinger and Springer, 1983). This molecule, termed LFA-1, is a protein dimer containing an alpha chain of 180,000 apparent molecular weight (Mr), which is noncovalently associated with a beta chain of 95,000 Mr. It is intriguing that LFA-1 shows structural homology with a second cell-surface protein, Mac-1, which mediates cell-cell interactions through recognition of cell-bound complement component C3bi. Mac-1 is also composed of two noncovalently associated polypeptides but in the case of Mac-1, the alpha chain is only 170,000 Mr.

Amino-terminal sequencing of the LFA-1 and Mac-1 alpha chains has demonstrated a low but significant degree of homology between the two. Based on these sequences, we have designed and are using oligonucleotide probes (synthesized by S. Horvath) to study the structure and genetic organization of the genes coding for these proteins. A similar approach is being taken in studies of the beta chains of the LFA-1 and Mac-1 molecules.

Reference:

* Dana-Farber Cancer Institute, Boston, MA.

28. EVOLUTIONARY CONSERVATION OF A CENTRAL NERVOUS SYSTEM-SPECIFIC GLYCOPROTEIN

Investigators: David B. Teplow, John W. Fabre*, William J. Dreyer

One approach to understanding the functional organization of the cells in the nervous system is to study cell-surface proteins whose expression and/or functions are unique to that system. Monoclonal antibodies are useful probes for these proteins. One such antibody, F3-87-B, has recently been described (Lakin and Fabre, 1981). The determinant identified by this antibody is present on a sialoglycoprotein found exclusively in the mammalian central nervous system (CNS). This protein has been found in brains of mice, rats, dogs and humans. It has not been detected in tissues outside of the CNS. The levels of expression of this protein increase as the brain matures during development.

Antibody F3-87-B has been used for affinity purification of the protein from both rat and human brains (Lakin et al., 1983). In both species the F3-87-B molecule migrated on SDS gels as a doublet whose members had apparent molecular weights of approximately 130,000 and 100,000. The levels of glycosylation and the amino acid compositions of the rat and human proteins were virtually...
identical. We have now determined the amino terminal sequences of these proteins in order to more precisely define their relatedness, to obtain basic information for computer assisted homology searches and structure/function analyses, and to provide sequences from which oligonucleotide probes may be designed for use in gene cloning.

Sixteen of the first 22 residues have been identified unambiguously in the proteins from both species. All 16 are identical. Of the remaining six residues, five are unidentified but, based on carbohydrate analysis, are likely to be glycosylated serines, threonines or asparagines. The sixth residue occurs at the amino terminus and appears to be identical in both molecules, but for technical reasons this conclusion must be considered tentative. These data convincingly demonstrate the extraordinary structural conservation of the f3-87-8 molecules through evolution and thus suggest that these sialoglycoproteins perform similar, crucial functions in these organisms. Structural and functional studies now continuing may define what these functions are.

References:

29. STRUCTURAL STUDIES OF THE MELANOMA-ASSOCIATED PROTEIN p97
Investigators: David B. Teplow, Joseph P. Brown*, William J. Dreyer

p97 is a cell-surface glycoprotein of 97,000 apparent molecular weight (M_r) which is expressed in high levels on cells of most human melanomas but only in trace amounts in normal tissues. As previously reported (Brown et al., 1982; Biology 1982, No. 37), the amino terminal protein sequence of p97 is homologous to that of plasma transferrin, the p97 molecule binds iron and a rabbit antiserum produced by immunization with pure p97 crossreacts with transferrin and lactotransferrin. To more fully characterize the structure of p97 and its organization in the plasma membrane, we have studied cleavage patterns produced after enzymatic proteolysis and have sequenced a number of the resulting peptide fragments.

Trypsin treatment of the purified protein produces a major N-terminal fragment of 45,000 M_r. This was proven by sequencing both unlabeled and 35S-cysteine-labeled peptides. In the case of the labeled peptide, the conservation of cysteines at positions 7 and 17, which is observed in transferrin, lactotransferrin and in conalbumin, was confirmed. Similar analyses of thrombin digestion products led to the identification of a 90,000 M_r N-terminal peptide. p97 thus appears to contain an N-terminal transferrin-like domain, in which iron binding presumably occurs, as well as a thrombin-sensitive site at the carboxy terminus which may represent a non-globular region of the protein adjacent to a portion involved in membrane penetration. These hypotheses are now being addressed through a combination of protein chemical studies and gene sequencing.

References:

Fred Hutchinson Cancer Research Center, Seattle, Washington.

30. PROTEIN SEQUENCE STUDIES OF BOVINE TRANSDUCIN
Investigators: David B. Teplow, James B. Hurley, Melvin I. Simon, William J. Dreyer

Information transfer in the vertebrate visual system is a complex process involving a cascade of interacting proteins and chemical mediators. Absorption of a single photon by rhodopsin in the rod outer segment (ROS) leads to the transient closure of some 10^6 sodium channels. This results in hyperpolarization of the ROS plasma membrane and conveyance at the synapse of the information that light has been detected.

For one photon to effect this process, a highly efficient system for signal amplification exists. The first amplification step in this system is the activation of an enzyme, transducin, by photolyzed rhodopsin. Transducin is a trimeric protein composed of an alpha chain (T_a) of 39,000 apparent molecular weight and a beta/gamma (T_b/g) subunit.
molecular weight ($M_r$), a beta chain ($\Gamma_\beta$) of 36,000 $M_r$ and a gamma chain ($\Gamma_\gamma$) of 6,000 $M_r$. We have initiated sequence studies of all three polypeptides to obtain basic structural information for use both in understanding the function of this enzyme and for providing suitable sequences for the design of synthetic oligonucleotide probes which will be used to clone the relevant genes.

The alpha, beta and gamma subunits of the enzyme have been purified to homogeneity by SDS-gel electrophoresis. Both the alpha and beta chains appear to have modified amino termini which are inaccessible to the sequencing chemistry. Peptides are thus being generated to provide reactive N-termini. We have identified 43 of 45 amino acids in the gamma subunit. Experiments are now in progress to identify the remaining two residues and to confirm the entire sequence. Computer assisted homology searching, performed by Russell Doolittle at the University of California in San Diego, has provided evidence that $\Gamma_\gamma$ is a novel polypeptide.

31. GENES RELATED TO RETROVIRUSES IN THE HUMAN GENOME

Investigators: Janet M. Roman, Carolyn Anderson, Elly de Pagter-Holthuizen

Our study of molecules that mediate specific cell-surface interactions during development has employed several experimental approaches (see previous abstracts). We believe that such molecules are highly polymorphic within an individual organism in order to allow for a large number of developmental options. It also seems likely that they are expressed in unique combinations on members of any one developmentally committed clone. Our previous work (Roman et al., 1981), based on studies of murine fibrosarcomas, revealed that a large family of cell-surface glycoproteins of 70,000 molecular weight (gp70 molecules) fulfilled both of these criteria. However, these proteins are closely related to the receptor proteins of retroviruses and the presence of a multitude of endogenous retroviral sequences in the murine genome makes these studies difficult to interpret. We therefore analyzed retroviral-related genes in the human genome, where no functional endogenous retroviral sequences have been detected.

We have screened a human DNA library with a 3.1 kb probe of baboon endogenous retroviral DNA containing the 3' portion of the polymerase gene, the entire gp70 (envelope) gene, the p15E gene, and the U3 region of the LTR (long terminal repeat). One clone derived from this screening, A9, has been shown to contain a 0.6 kb portion which hybridizes with the baboon LTR, and other portions which hybridize only weakly at low stringencies with other portions of the baboon retrovirus genome. We have determined that the 0.6 kb LTR-related sequence is present in multiple copies in the human genome. We have found no evidence for its somatic rearrangement in comparisons of blots of DNA from sperm versus adult tissues. Also, we have not found it to be concentrated in satellite DNA, which had been hypothesized for retroviral LTRs in the case of bovine satellite DNA (Streeck, 1981).

Recently, other laboratories have also found evidence for retrovirus-related genes in humans (Bonner et al., 1982; Noda et al., 1982; Repaske et al., 1983). We conclude that the human genome contains many genes related to retroviruses. Whether these genes function either in normal development or in disease is unknown.

References:

PUBLICATIONS


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Summary: Our laboratory is interested in dissecting the nature of the vertebrate immune response. To this end, we are studying several families of genes that encode various aspects of immune responsiveness such as the antibody genes, the genes of the major histocompatibility complex and certain growth hormone genes (colony-stimulating factors) that regulate the development of lymphocytes involved in immune responses. Our model system is the inbred mouse. We use technical approaches that include protein chemistry, immunology and genetic engineering. Our laboratory also has been involved in developing microchemical instrumentation that allows us to manipulate genes and proteins more effectively. Recently, we have become interested in the relationship between oncogenes and growth factor as exemplified by the striking homology relationship between the serum growth factor, platelet-derived growth factor and the oncogenes v-sis.

The genes of the major histocompatibility complex (MHC) fall into two categories denoted class I and class II (Hood et al., 1983). Both facilitate the ability of the immune response to distinguish self from non-self. Class I genes, typified by transplantation antigen gene products, are made up of a
transmembrane heavy chain of 45,000 daltons consisting of three external domains, a transmembrane region and a cytoplasmic domain, noncovalently associated with β₂-microglobulin, a small polypeptide demonstrating homology to the constant regions of immunoglobulins. We have cloned approximately 40 distinct class I genes from the inbred BALB/c mouse and determined their approximate position within the major histocompatibility complex that spans approximately 3000 kilobases of DNA on chromosome 17 of the mouse. At least 20 of these class I genes are expressed in gene-mediated transfer experiments into mouse L cells. We have started a series of in vitro mutagenesis and gene transfer experiments to begin dissecting the function of these gene products (Zuniga et al., 1983).

Six class II genes have been isolated from BALB/c mice and two functional alpha (Aα, Eα) and two functional beta (Aβ, Eβ) have been identified in approximately 250 kilobases of contiguously cloned genomic DNA. We have sequenced the Aβ and Eα genes and portions of them show striking homology relationships to one another, to their class I counterparts and to antibody genes. We have succeeded in DNA-mediated gene transfer of class II genes in the mouse L cells. We are attempting to develop retroviral gene transfer systems for the transfection of MHC genes into lymphocytes and plan to carry on in vitro mutagenesis studies with class II genes. One particular observation of striking interest is our inability to find a gene encoding a suppressor polypeptide (I-J) that immunogenetic studies suggest should fall in the middle of the class II region.

Our studies on antibody genes have taken two directions. First, we are studying the evolution of the small T15 VH gene that contains four genes. We have isolated and sequenced these genes from two inbred strains of mice and we are in the process of isolating these genes from several subspecies of mice. Second, we are studying the oncogene, c-myc, which generally translocates from chromosome 15 to the heavy chain gene chromosome 12 in most mouse plasmacytomas (Crews et al., 1983). We also have studied several other rearrangements of oncogenes into the antibody gene families. The activation of these oncogenes by their translocation into antibody gene families raises fascinating questions about the regulation and function of oncogenes.

We have employed a variety of approaches to attempt to clone the genes encoding the T-cell receptor. These approaches have clearly established that the T-cell receptor is not encoded by immunoglobulin VH genes. Our current efforts are directed at attempting to clone the I-J gene through the use of a bacterial expression system and monoclonal anti-I-J antibodies.

Our laboratory has recently begun to study the amino acid sequence of a series of hormones, including human platelet-derived cofactor, rat and mouse transforming growth factors, and mouse colony-stimulating factors. The platelet-derived growth factor shows a striking homology to the woolly monkey oncogene v-sis (Doolittle et al., 1983). Thus, this oncogene may be a virally controlled cell growth factor which can only act on those classes of cells which have the appropriate receptor for this growth factor. The platelet-derived growth factor stimulates a variety of connective tissue cells in conjunction with the healing of wounds. Thus, one would predict that the v-sis shared the transformed cells of connective tissue types, and studies to investigate this hypothesis are now under way. This observation raises a variety of intriguing possibilities for immunologic therapeutic approaches to the control of cancer. We also plan to study the receptors for several of these hormones.

We have moved gradually toward molecular neurobiology through the cloning of the gene encoding the rat myelin basic protein (Rosch et al., 1983). This gene has been used to demonstrate that in a mouse neurologic mutant denoted shiverer, portions of the gene encoding myelin basic protein are deleted. This gene should permit us to study a variety of demyelinating and dysmyelinating diseases in man and mouse including multiple sclerosis.

Our laboratory has continued the development of microchemical instrumentation this past year (Hunkapiller et al., 1983). We have finished the development of an automated DNA synthesizer with a
10-minute cycle time, the ability to synthesize 40-mer DNA probes, and the ability to synthesize heterogeneous probes. We have now under development an automated peptide synthesizer and an automated DNA sequenator.

Thus, our laboratory has the ability to focus a variety of immunological, protein chemical, and nucleic acid techniques in conjunction with powerful microchemical instrumentation on fundamental problems of developmental biology.

References:

32. THE MOLECULAR GENETICS OF MURINE ANTI-STREPTOCOCCAL ANTIBODIES

Investigators: Roger M. Perlmutter, Joan L. Klotz*, Martha W. Bond**, Moon Nahm+, Joseph M. Davie+

Higher vertebrates possess an almost unlimited repertoire of different antibody specificities that form the cornerstone of the immune system. In order to analyze the genetic mechanisms responsible for the generation of antibody diversity, we have focused our attention on the murine immune response to group A streptococcal carbohydrate (GAC). We have previously shown that individual mice produce only one or at most a few antibodies in response to immunization with GAC vaccine even though the repertoire of the entire mouse strain for this antigen is quite large. In addition, serologic and isoelectric focusing analyses of hybridoma anti-GAC antibodies from the A/J mouse strain suggest that a common light chain is employed in more than 50% of A/J anti-GAC antibodies. Using protein and nucleic acid sequencing techniques, we hoped to assign the observed diversity in the GAC system to mechanisms involving multiple germline elements, combinatorial joining of these elements, combinatorial association of heavy and light chains and/or somatic mutation acting on a limited number of germline gene segments.

Amino-terminal sequence analysis of four heavy and five light chains from anti-GAC antibodies confirms that these molecules are highly restricted; the heavy chains differ at only two positions while the light chains fall into two different sequence groups, previously defined by a light chain-specific idiotype, with three idiotype-positive molecules sharing an identical sequence through residue 40. We have now cloned one of the V_H genes responsible for A/J anti-GAC antibodies and find that it includes a V_H gene segment that is 97% homologous to a previously defined V_H element which directs the synthesis of anti-inulin heavy chains in BALB/c mice. A careful analysis using coding region and flanking region probes demonstrates that the murine genome includes multiple closely homologous V_H gene segments which can participate in the formation of anti-GAC antibodies. In addition, these V_H segments have undergone very recent gene duplication events in different mouse strains. Thus, in contrast to results obtained using antibodies directed against phosphorylcholine, we find that the generation of diversity in antibodies to GAC depends in part on the utilization of multiple germline genes.

33. SEQUENCE ANALYSIS OF THE T15 V_H GENE FAMILY

Investigators: Gerald J.-M. Siu, Stephen T. Crews, Elizabeth Springer, Kathryn Calame*

We are interested in studying the structure, evolution, and expression of the heavy chain variable region (V_H) gene locus. Since the mouse genome contains hundreds of V_H genes, we have focused on a small family of four closely related genes as a model system, called the T15 V_H gene family (Crews et al., 1981). Large stretches of DNA flanking the four genes have been sequenced using the dideoxy method and the chemical cleavage procedure of Maxam and Gilbert. This information will hopefully allow a more complete understanding
of the structure of \( V_H \) genes, identification of potentially important regions for transcription control, and a more complete understanding of the molecular events involved in the evolution of these genes. Using \( S_1 \) nuclease mapping, one of us (K.C.) has identified the initiation point of transcription as being 64 bp 5' to the leader exon (Clarke et al., 1982). Sequence analysis of the immediate 5' flanking regions has identified consensus sequences which have been implicated in the control of transcription. Additional regions of \( V_H \) gene-specific homology have been found which also may be important in gene expression. Heteroduplex analysis of the members of this family in Dr. Calame's lab reveals extensive flanking region homology; sequence analysis of the regions where homology ends is currently under way in the hope that this would allow a better understanding of the mechanism of gene duplication.

We also have analyzed the members of this family utilizing the technique of Kimura (1981) to study the time periods of divergence and the mutation rates of the coding, intron, and flanking regions. We have found that the four genes have diverged from each other at time periods ranging from 1 to 13 million years ago. Unexpected discrepancies in the calculated divergence times between two of the members (utilizing two techniques) may indicate a coding region gene conversion event. Calculations of mutation rates indicate that the mutation rate in the second codon position is significantly higher than the mutation rate in the first codon position; this is not consistent with other genes (Miyata et al., 1980; Kimura, 1981). We hope that a complete study of the mutation rates and the codon usage of these genes will allow a better understanding of the way in which these genes are evolving.

References:
*Department of Biological Chemistry, University of California, Los Angeles.

34. CHROMOSOMAL ARRANGEMENT OF ANTIBODY VARIABLE REGION GENES

Investigators: Gerald J.-M. Siu, Stephen T. Crews, Elizabeth Springer

The antibody heavy chain gene locus contains hundreds of heavy chain variable region (\( V_H \)) genes scattered over a large region of chromosome 12 of the mouse. An understanding of the function, evolution, and expression of these genes requires a knowledge of the physical arrangement of these genes along the chromosome. We have chosen the T15 gene family in the BALB/c mouse as a model system to study this question. The four members of this family (designated V1, V3, V11 and V13) have been isolated from a recombinant lambda library and sequenced (Crews et al., 1981). A cosmid library has been constructed and screened with the T15 probe. Recombinant cosmids containing the four genes have been isolated and analyzed. V1 and V3 were found to be separated by 15 kb of DNA; V11 and V13 were each found to be amidst 33 kb of DNA that is unlinked to any other \( V_H \) gene. Experiments have shown that our hybridization conditions can detect all known \( V_H \) genes; thus we may exclude the possibility of distantly related \( V_H \) genes also residing on these clones. Using cloned repeat probes, we have identified the presence of the repeat element MIF-1 near three of the four genes. Utilizing further experiments with the cosmid library, we hope to eventually link all four members of the family and map the presence of other \( V_H \) genes, repeats, and other elements to determine the complete picture of the linkage and organization of this family and of the \( V_H \) gene family in general.

Reference:

35. STUDIES ON A LARGE \( V_H \) FAMILY IN BALB/c MICE

Investigators: Donna L. Livant, John B. Wall*

Dextran is the branched, storage polysaccharide found in bacteria that contains \( \alpha-1,3, \alpha-1,4 \) and \( \alpha-1,6 \) linked glucose in varying proportions (Sugii et al., 1981). Protein sequence analysis of heavy chain variable regions from 21 BALB/c hybridomas
has shown that when a BALB/c mouse is immunized with the α-1,3 dextran, B13555, it expresses at least five different V\textsubscript{H} segments in its immune response (Clevinger et al., 1981). One of these V\textsubscript{H} segments corresponds to that of the myeloma antibody J558 and was found in 16 of the 21 hybridoma antibodies sequenced. We have, therefore, defined the J558 V\textsubscript{H} gene as the prototype gene for the J558 gene family.

In order to isolate the J558 V\textsubscript{H} gene in its germline configuration, we have screened a sperm library from BALB/c and isolated 97 clones that hybridize to the J558 gene. Half of these clones contain at least one V\textsubscript{H} gene. The remainder contain at least two or three V\textsubscript{H} genes. We are currently studying the set of these clones having at least one V\textsubscript{H} gene with significant 5\textsuperscript{'} flanking sequence homology to that of the J558 gene (Biology 1982, No. 43). We would like to know whether any of these V\textsubscript{H} genes from the germline correspond to those expressed in the BALB/c response to α-1,3 dextran.

References:

*Undergraduate, California Institute of Technology.

36. THE EVOLUTION OF THE T15 GENE FAMILY IN MUS

Investigator: Gerald J.-M. Siu

I am interested in studying the evolution of the V\textsubscript{H} gene family. It is believed that the V\textsubscript{H} gene family encompasses over 300 genes and that individual V\textsubscript{H} genes can differ by as much as 60% on the DNA level. This family presents a unique problem in gene maintenance; how does the V\textsubscript{H} gene family manage to maintain its diversity and size? I hope to investigate this question by studying homologous V\textsubscript{H} genes in different species of Mus. Using the T15 probe, genomic blots utilizing DNA from six different species of Mus have been analyzed. Each has between 5 to 12 hybridizing bands, indicating that the T15 Family has undergone distinct duplication events in different evolutionary lines in a relatively short period of time. A recombinant lambda phage library has been constructed utilizing DNA from Mus caroli, one of the species analyzed, which has five major hybridizing bands. The library was screened with the T15 probe, and 26 recombinant phage have been isolated and are currently being analyzed. These V\textsubscript{H} genes from M. caroli that hybridize to the T15 probe will be subcloned and sequenced. Utilizing the method of Kimura (1981) mentioned above in conjunction with the fossil record, I hope to determine the history of the T15 family in Mus, which will hopefully provide insight on the molecular mechanisms of evolution in the V\textsubscript{H} gene family.

Reference:

37. RECENT EVOLUTION OF THE T15 GENE FAMILY IN MICE AND RATS

Investigators: Roger M. Perlmutter, Bennett J. Berson, Johanna A. Griffin*

Considerable insight into the genetic mechanisms underlying the generation of antibody diversity has been gained through analysis of murine antibodies specific for phosphorylcholine (PC). Previous work in this laboratory has established that a single germline V\textsubscript{H} gene segment, called V1, directs the synthesis of essentially all anti-PC heavy chains in BALB/c mice. Three additional closely-related V\textsubscript{H} segments, V3, V11 and V13, were cloned and sequenced from a library of BALB/c genomic DNA and together these four elements (including V1) comprise a group of sequences greater than 88% homologous referred to as the T15 gene family. Research in other laboratories has established that anti-PC antibodies are highly protective against challenge with live pneumococcal organisms, suggesting that the V1 gene segment is probably an important part of the murine germline antibody repertoire. The V3 gene segment, on the other hand, is a pseudogene and cannot encode a functional polypeptide. Thus the T15 gene family provides an ideal substrate for the study of evolutionary pressures in multigene families since one element, V1, is presumably subjected to strong selection pressures while another element, V3, is a
pseudogene and should drift in the absence of significant selective forces.

Detailed structural analysis of the T15 gene family in a very different mouse strain, B10.P, and in inbred rats confirms these hypotheses. The V1 gene segment of B10.P mice is 98% homologous to the V1 gene segment of BALB/c mice and the coding regions are more conserved than are the intervening sequences. Conservation of the V11 and V13 sequences is also apparent. In contrast, the V3 gene segment of B10.P mice is 95% homologous to its BALB/c counterpart and has diverged at a rate comparable to the intervening sequences. Southern blot analysis reveals that the T15 gene family has undergone significant expansions and contractions during the approximately 10 million years since the divergence of rats and mice. These studies demonstrate that even though somatic mutational mechanisms may greatly alter the expressed repertoire of V_H genes, specific germline V_H gene segments are of fundamental importance in the generation of antibody diversity.

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38. AN ANALYSIS OF THE EXPRESSION OF IMMUNOGLOBULIN HEAVY CHAIN VARIABLE REGION GENE SEGMENTS BY T CELLS

Investigators: Ellen B. Kraig, Mitchell Kronenberg

An organism can respond to many diverse antigens, yet individual T and B lymphocytes synthesize receptors that recognize only a restricted set of antigenic determinants. There is considerable controversy as to whether any of the gene segments encoding immunoglobulin, the receptor for antigen on B cells, are also used to encode part of the T-cell antigen receptor. It was shown that T cells fail to rearrange or transcribe productively any of the immunoglobulin joining or constant region gene segments. However, a large amount of serological and genetic data exists that suggests the expression of immunoglobulin heavy chain variable (V_H) regions by T cells. Using two different molecular approaches, we have analyzed the expression of V_H gene segments by T cells.

First, we have attempted to determine whether B and T cells, which have the same antigenic specificity and whose receptors share idiotypic determinants, will in fact transcribe very similar V_H gene segments. We have chosen to examine the immune response to GAT (a synthetic polypeptide of glu_60, ala_30, tyr_10), because T cells and B cells specific for GAT have been cloned and they share a predominant idiotypic determinant, CGAT. We have obtained and characterized a cDNA clone containing the entire coding sequence for the V_H gene from a GAT-binding immunoglobulin. The GAT-V_H clone was hybridized to Northern blots containing RNA from 13 different GAT-specific T hybridomas; there was no evidence of a T-cell transcript that hybridized to the GAT-V_H probe. (The GAT-specific T cells were provided by Drs. J. Kapp, C. Pierce, A. Abruzzini and C. Sorensen [Jewish Hospital, St. Louis] and L. Samelson and R. Schwartz [NIH, Bethesda].) We conclude that T and B cells responding to GAT do not utilize similar V_H gene segments.

Second, we wished to determine whether T cells transcribe any V_H gene segment from the B cell repertoire. Using RNA from T-cell hybridomas specific for three different antigens, we constructed large cDNA libraries. (The T-cell hybridomas were kindly provided by Drs. P. Marrack and J. Kappler [National Jewish Hospital, Denver] and J. Kapp and C. Pierce [Jewish Hospital, St. Louis].) These libraries first were screened with two probes: 1) a synthetic oligonucleotide complementary to the RNA that encodes a highly conserved portion of the immunoglobulin V_H gene segment, and 2) a cDNA synthesized from spleen RNA using specific priming. Either probe will be able to hybridize to most of the known V_H gene segments, yet neither is completely specific for V_H sequences. However, a T cell cDNA clone which hybridized to both probes would probably be a V_H gene. There were no such T cell cDNA clones. In addition, the T cell cDNA libraries were screened at low stringency with two V_H gene segments. These were chosen because one or the other shares at least 55% sequence similarity with every sequenced murine V_H gene. However, no T-cell clones hybridized to the V_H probes. Therefore, we conclude that the T cell antigen receptor is not encoded by V_H gene segments.
MOUSE c-myc ONCOGENE IS LOCATED ON CHROMOSOME 15 AND TRANSLOCATED TO CHROMOSOME 12 IN PLASMACYTOMAS

Investigators: Richard K. Barth, Kathryn Calame*, Stephen T. Crews, Stuart K. Kim, Naoki Takahashi

Characteristic chromosomal translocations are often associated with lymphoid tumors in both mice and humans. It has been postulated that these abnormalities may contribute to the generation of tumors by altering the expression of certain cellular genes, referred to as oncogenes. Therefore, investigation of the nature of tumor-associated chromosome translocations at the molecular level may help to elucidate the mechanism of oncogenesis.

We have been studying a chromosomal abnormality characteristic of mouse plasmacytomas, a tumor derived from antibody-producing cells. Plasmacytomas frequently possess a reciprocal translocation between chromosomes 12 and 15 (Ohno et al., 1979). Previously in this laboratory, genomic clones were isolated from recombinant lambda libraries constructed from the plasmacytomas MOPC167 and MOPC603. Among these were clones which spanned the chromosome 12/15 translocation junction of these tumors (Calame et al., 1982). Analysis of these clones reveals that at the site of translocation, the antibody-heavy chain gene (Ca), located on chromosome 12, has been juxtaposed to the c-myc gene, located on chromosome 15 (Crews et al., 1982). The c-myc gene is the cellular homologue of the transforming gene of the avian retrovirus MC29. The nature of the translocation is such that the Ca gene is positioned in the opposite transcriptional orientation as the c-myc gene.

The structures of the rearranged and unarranged c-myc genes have been compared by restriction enzyme mapping and DNA sequencing. The c-myc gene is comprised of three exons which span 4.8 kb. The first exon is composed exclusively of 5'-untranslated sequences. Translation initiates 60 nucleotides into the second exon. In MOPC167 and MOPC603, chromosome translocation occurs within the first intron, approximately 1.1 kb before the second exon. Therefore the translocation has eliminated the first exon as well as the normal transcriptional control signals from the rearranged gene. Transcription of the aberrant c-myc gene initiates at multiple sites within the first intron, resulting in shortened transcripts. However, there is no evidence for the synthesis of altered c-myc polypeptides from these truncated mRNAs.

Transfer of DNA isolated from mouse plasmacytomas to the NIH3T3 fibroblast cell line results in the formation of neoplastically transformed foci. We have shown that the rearranged c-myc gene is not responsible for this event. However, Weinberg and coworkers have demonstrated that transfer of the rearranged c-myc gene is a prerequisite for the successful transformation of primary mouse embryo fibroblasts by a number of oncogenes which transform NIH3T3 cells. Thus, mutation of the c-myc gene appears to be one of a number of events that serve to participate in the generation and/or maintenance of the transformed phenotype of plasmacytomas.

References:
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CHARACTERIZATION OF A NOVEL CHROMOSOMAL TRANSLOCATION EVENT IN A MURINE PLASMACYTOMA

Investigators: Roger M. Perlmutter, Joan L. Klotz*, Bennett J. Berenson

Specific chromosomal abnormalities are regularly associated with many murine and human malignancies. In particular, the majority of murine plasmacytomas contain a characteristic 12:15 translocation which results in the juxtaposition of a cellular oncogene, c-myc, with the immunoglobulin heavy chain gene locus, and an analogous rearrangement event has been observed in human Burkitt's lymphomas. The rearranged c-myc in BALB/c mouse plasmacytomas directs the synthesis of qualitatively and quantitatively abnormal transcripts which may play an
etiologic role in the development of the transformed state in these lymphoid malignancies. In the course of an ongoing investigation into the relationship between DNA rearrangements and oncogenesis, we have identified a novel 6:10 chromosomal translocation in the murine plasmacytoma cell line NS-1 which juxtaposes the immunoglobulin Cκ gene with a single-copy sequence of unknown function that is strongly conserved in all vertebrate species. The NS-1 plasmacytoma is a frequently-employed fusion partner in hybridoma production and is known to contain a rearranged c-myc gene. Several important features of the 6:10 translocation in NS-1 cells have become manifest:

1) Translocations involving the intervening sequence 5' to Cκ may occur by a site-specific mechanism directed towards a region about 1 kb from the Cκ gene.

2) The chromosome 10-derived portion of this translocation, which we call TκNS-1, is very actively transcribed in NS-1 cells but in no normal adult tissue.

3) At least one other malignant B cell line contains a rearranged copy of TκNS-1.

We conclude that individual B cell tumors may contain multiple chromosomal aberrations perhaps relevant to oncogenesis.

Our current work is directed towards a more detailed analysis of the structure and function of the TκNS-1 element.

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41. CLASS I GENES IN THE H-2K SUBREGION IN BALB/c MICE

Investigator: Beverly Taylor Sher

The major histocompatibility complex of the mouse, or H-2 complex, encodes a variety of molecules that function in the immune response. The H-2 complex can be divided into four subregions, K, I, S and D. The K and D subregions encode the heavy chains of the transplantation antigens, which are class I molecules. These molecules function in the control of the cellular immune response to viral antigens.

The transplantation antigen genes belong to a heterogeneous multigene family. The BALB/c mouse, which is of the H-2K haplotype, expresses three different transplantation antigen molecules that have been isolated and partially sequenced: Kd, Dd, and Ld. Dd and Ld both map to H-2D; Kd maps to H-2K. In addition, there is serological evidence that the H-2D subregion in BALB/c mice may encode other transplantation antigens, M<sup>d</sup> and R<sup>d</sup> (Iványi and Démant, 1979; Hansen et al., 1981). We find no evidence for these genes by cloning and gene transfer experiments. It appears that L molecules are expressed by mice of only two haplotypes, H-2<sup>d</sup> and H-2<sup>q</sup>, while D molecules are expressed by all haplotypes.

Clones containing genes encoding D<sup>d</sup> and L<sup>d</sup> have been isolated from genomic libraries (Moore et al., 1982; Steinmetz et al., 1982). The DNA sequence of the L<sup>d</sup> gene has already been determined. The DNA sequence of the D<sup>d</sup> gene is being determined by the dideoxy method. In addition, a gene whose coding region has an identical restriction map to that of the D<sup>d</sup> gene but whose flanking sequence differs has been shown by gene transfer experiments to encode molecules bearing D<sup>d</sup> determinants (R. S. Goodenow, personal communication). This gene is being mapped by restriction enzyme polymorphisms using a low-copy probe. If it maps to H-2D, its sequence also will be determined. Comparison of the sequences of the functional genes in the H-2D subregion of BALB/c mice should help to clarify the evolutionary history of this subregion. Comparison of these sequences with those of genes from the H-2K subregion of BALB/c as well as with those of transplantation antigen genes from other haplotypes should help reveal the nature of the processes leading to the differences in gene number and the striking polymorphism characteristic of this heterogeneous multigene family.

References:
Iványi, D. and Démant, P. (1979) Immunogenetics 8, 539-545.
42. STUDY OF THE MOLECULAR BASIS OF TWO MOUSE TRANSPLANTATION ANTIGEN MUTATIONS

Investigator: Yi Henry Sun

Mouse strains with mutations in their transplantation antigen are important in providing valuable information on the link between biological function and molecular structure. Most mutants carry one or more point mutations causing amino acid substitutions. The two mutants under study here are unusual in that their mutations seem to be more extensive. Both the L and D molecule produced by the dm1 mutant mouse are structurally and serologically different from those produced by the parent B10.D2 (H-2^d) haplotype mice (Brown et al., 1978; Wilson et al., 1982). The dm2 mutant produces no L molecule (McKenzie et al., 1977) in contrast to its parental BALB/c strain (H-2^d haplotype).

Low copy-number DNA sequences flanking the L^d gene were isolated from cosmid clones of a BALB/c sperm DNA library (Winoto et al., 1983; Y. H. Sun, unpublished). These sequences were used as probes in Southern blots of liver DNA digested with various restriction enzymes. Two 5' flanking probes showed that the corresponding restriction fragment is absent in both mutants, while the 3' flanking probe detected no difference from the parent strains. This demonstrates that the mutations are caused by DNA rearrangement or deletion, and defines one of the breakage points to be within the 18 kb region between the 5' and the 3' flanking probes.

To compare the structural organization of this region between the mutants and their parent strains, cosmid cloning is the ideal approach. I constructed a dm1 cosmid library and, using the set of probes described above, isolated 14 overlapping clones which span 82 kilobases. This region contains one class I gene. When the restriction sites are compared with those of the cloned BALB/c L^d region, it is found that the 3' region of the mutant gene is identical to the L^d region, but differs in the immediately 5' region. The precise location of the breakage point awaits further refined mapping. Fine mapping of the 5' flanking sequence will tell us whether the promoter is affected and transformation experiments will show whether this gene is functional.

References:

43. DNA SEQUENCE ANALYSIS OF THE Tla GENES OF THE BALB/c MOUSE

Investigator: Douglas A. Fisher

Thymus leukemia (TL) antigens are cell-surface glycoproteins that are normally expressed only on thymocytes of TL^+ mice (Vitetta and Capra, 1978). Seven antigenic specificities have been defined. Leukemias of TL^+ mice may express the thymocyte TL specificities, or may express new TL antigens. TL antigens also are found on leukemias of thymic origin in TL^- mice.

Histocompatibility antigens (H-2), the T-cell differentiation antigens Qa1 and Qa2, and TL antigens are all structurally similar in that they consist of two 45,000 molecular weight (MW) polypeptide chains noncovalently associated with two molecules of the same 12,000 MW subunit, β2-microglobulin. The genes encoding these antigens also are all closely linked on mouse chromosome 17.

Recently, two genomic clones isolated from a lambda phage genomic library by hybridization with cloned H-2 cDNAs were shown to produce TL antigens when transformed into mouse L cells (Goodenow et al., 1982). We have begun DNA sequence analyses of these clones with the aim of defining the gene structure of TL antigens and clarifying the relationship between H-2 and Tla genes. The Tla system may prove to be an excellent system in which to study gene expression, since the genes are expressed normally in limited cell types as well as aberrantly in leukemia cells. In addition, TL expression may be regulated in cell culture in response to thymic hormones (Goldstein et al., 1980). With TL-specific probes isolated from Tla genes, unlike the crossreacting H-2 probes, it should be possible to analyze TL genes and their
RNAs in normal and leukemic cells, as well as in different haplotypes of mice.

References:

44. NUCLEOTIDE SEQUENCE OF AN A6 GENE AND HOMOLOGY WITH MAJOR HISTOCOMPATIBILITY COMPLEX AND IMMUNOGLOBULIN GENES

Investigators: Marie Malissen, Tim Hunkapiller

The major histocompatibility complex (MHC) of the mouse is a cluster of genes encoding different classes of proteins involved in immune responses. Class II products are cell-surface glycoproteins required for antigen presentation and lymphocyte interactions. They are composed of two noncovalently associated chains designated \( \alpha \) (34,000 daltons) and \( \beta \) (28,000 daltons). We have determined the entire nucleotide sequence of an A\( ^d \) gene isolated from a BALB/c cosmid library (Steinmetz et al., 1982). The presence of six exons was predicted by comparison with the cDNA sequences of their human counterparts and with partial protein data.

The genomic organization of this gene and its sequence has been compared with other genes involved in immune response (class I genes, class II genes, \( \beta_2 \)-microglobulin genes, and antibody genes). It appears from the sequence homology observed that all these genes can be included in a superfamiliy. Analysis of sequence homology and genomic organization allowed us to presume an evolutionary relationship of these genes and particularly to suspect that class II and class I polypeptides may be derived from a prototypic ancestor with two chains (Malissen et al., 1983).

References:
46. EXPRESSION OF MOUSE CLASS II
HISTOCOMPATIBILITY ANTIGENS IN L CELLS
AFTER DNA-MEDIATED GENE TRANSFER

Investigators: Bernard Malissen, Michael Steinmetz,
Minnie McMillan, Michel Pierres*

Mouse Ltk- cells were cotransformed with the HSV
 tk gene and various combinations of cosmid clones
 containing H-2 class II genes isolated from either
 a BALB/c (H-2d) or an AKR (H-2k) genomic library.
Uncloned tk+ transformants selected in HAT medium
 were analyzed with anti-class II monoclonal anti­
 bodies (mAb) by a two-site sandwich immunoassay.
This assay allowed the detection of class II
 molecules localized both at the cell surface and in
 the cytoplasm. Positive cell populations were
 confirmed by RNA blot analysis and subsequently
 cloned by limiting dilution. The cloned tk+ L
 cells transfected with both the Ak and the Ak genes
 consistently expressed Ak molecules detectable by
 mAb directed against the A\(\alpha\)A\(\beta\) dimer. Serological
 analysis performed with a panel of 12 mAb indicated
 that these molecules are indistinguishable from the
 ones produced by C3H spleen lymphocytes. The Ak
 products expressed by the transformants were
 biosynthetically labeled, isolated by immuno­
 precipitation and analyzed by two-dimensional gel
 electrophoresis. The positions of the Ak\(\alpha\)A\(\beta\) spots
 are identical to those observed with Ak molecules
 precipitated from C3H splenocytes. However, no
 molecular species migrating at the position of the
 invariant chain (\(\iota\)) spot appeared to be present in
 the Ak-positive cells. Ak-positive L cells and C3H
 spleen cells contained similar amounts of Ak
 molecules when assayed using the two-site sandwich
 immunoassay. The cell surface expression of Ak by
 the L cell transformants also is similar to that of
 C3H spleen cells. Gene transfer experiments have
 demonstrated that the \(\iota\) gene product is not
 necessary for obtaining an efficient transport of
 class II \(\alpha\) and \(\beta\) chains to the cell surface.

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48. EXPRESSION AND FUNCTION OF TRANSPLANTATION
ANTIGENS WITH ALTERED OR DELETED
CYTOPLASMIC DOMAINS

Investigators: Martha C. Zuniga, Bernard Malissen,
Minnie McMillan, Peter R. Brayton*

The cytoplasmic domain of the class I trans­
plantation or histocompatibility antigen is encoded
on four exons (part of exon 5 and exons 6, 7, and
8). The amino acids encoded by the fifth and sixth
exons are highly conserved, whereas those of the
seventh and eighth exons are more variable. Recent
comparisons of protein, cDNA and genomic sequences
have led to the conclusion that differential
splicing occurs between the seventh and eighth
exons, giving rise to some of the diversity in this
region. These observations together with the
identification of conserved residues which can
serve as phosphorylation sites and of a conserved
cysteine residue have prompted speculations about
the functions of cytoplasmic domains of trans­
plantation antigens. It has been proposed that the
cytoplasmic domain functions in transmembrane
signalling, perhaps by interaction with the
cytoskeletal matrix. In addition, class I
molecules might form covalent dimers or interact
with other proteins via the conserved cysteine
residue. Finally, the sequence diversity
contributes by differential splicing at the seventh
intron introduces the possibility that this region
of the molecule interacts with viral antigens
during virus-restricted killing. An interaction
between the class I antigen cytoplasmic domain and
adenovirus antigen has recently been suggested.

To directly address the question of cytoplasmic
domain function, we have used in vitro mutagenesis
to alter or delete the cytoplasmic domain of a
cloned transplantation antigen gene, the 27.5 Ld
gene. Two mutants of this gene have been
constructed. In one mutant the cytoplasmic domain
of the class I molecule has been altered by
deletion of 24 of the 31 C-terminal residues and in
the second the C-terminal 25 residues of the cyto­
plasmic domain have been replaced with a unique
sequence of 19 amino acids. These mutant class I
genes have been transferred into mouse L cells by
DNA-mediated gene transfer. Both mutant genes are
expressed at normal levels on the cell surface, and
they have charge properties and sizes consistent
with the introduced alterations. These mutant Ld
molecules can serve as target antigens for allo­
genetic cytotoxic T cells and as restricting
elements for virus-specific cytotoxic T cells.
These results show that the 24 residues replaced or
deleted from the carboxy terminus of the class I molecule are not required for its transport to or integration in the plasma membrane, nor for its function as a target antigen or a restricting element during T cell-mediated cytotoxicity.

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49. IDENTIFICATION OF SEQUENCES IMPORTANT FOR INTERCELLULAR TRANSPORT AND MEMBRANE INTEGRATION OF A TRANSMEMBRANE GLYCOPROTEIN BY IN VITRO MUTAGENESIS

Investigators: Martha C. Zuniga, Suzanna J. Horvath

Class I histocompatibility antigens are integral membrane glycoproteins present on the surfaces of virtually all nucleated cells of a mammal. The 45,000-dalton class I polypeptide shares several structural features with other transmembrane proteins. Among these are the 24-residue uncharged and hydrophobic transmembrane segment and the basic residues immediately following the hydrophobic segment. The basic residues have been postulated to interact with the negatively-charged phospholipid groups on the inner face of the membrane and to thereby fix the protein in the membrane. Alternatively, these residues might serve as signals for intracellular transport of transmembrane proteins.

To determine the function of the basic residues in the intracellular transport and localization of transmembrane proteins, we are employing in vitro mutagenesis of a cloned class I histocompatibility antigen gene, the Ld gene of the BALB/c mouse. This gene has eight exons, the fifth of which encodes the hydrophobic transmembrane segment and residues lys-arg-arg-arg-asn-thr, which immediately follow the hydrophobic segment. The basic residues have been postulated to interact with the negatively-charged phospholipid groups on the inner face of the membrane and to thereby fix the protein in the membrane. Alternatively, these residues might serve as signals for intracellular transport of transmembrane proteins.

Since BAL31 digestion can be strictly controlled, this strategy should generate a variety of mutant Ld genes whose products range from those having no basic residues at the end of the transmembrane segment to those having one or more basic residues and from those having no acidic residues at the end of this region to those having acidic residues either alone or in combination with remaining basic residues. The expression of the mutant Ld genes will be examined after introduction into mouse L cells and the cellular distribution of the mutant Ld gene products will be determined with anti-Ld monoclonal antibodies. Hence we should be able to assess the effect of the nature of charge groups on residues immediately following the transmembrane segment on the transport and/or membrane integration of the Ld polypeptide.

50. STRUCTURE-FUNCTION STUDIES ON MURINE HISTOCOMPATIBILITY ANTIGENS

Investigators: Iwona Stroynowski, Robert S. Goodenow, Susan G. Schiffer, Anders Orr

A major goal of our studies on H-2 genes is to elucidate the relationship between the structure of molecules encoded in the murine major histocompatibility complex and their functional interactions with other molecules. In particular, it is important to identify the regions on H-2 antigens that interact with the T-cell receptor during allo- geneic T-cell killing and in H-2 restricted killing of virally infected cells by cytotoxic T cells. We also are interested in mapping polymorphic determinants on class I molecules, which are recognized by specific antibodies and in their relation to the structural determinants recognized by T-cell receptors. In an effort to answer these questions, we have generated recombinant genes using standard molecular manipulations on genes cloned and sequenced in this laboratory (Goodenow et al., 1982a; Steinmetz et al., 1982). We have constructed "shuffled" genes which encode defined class I hybrid molecules by exchanging homologous sets of exons (exons 1-3) between H-2Ld, H-2Dd and H-2Kd genes. These genes were cotransformed with the herpes simplex virus thymidine kinase gene (tk) into tk- mouse L cells (H-2Kd). After selection in HAT medium (Goodenow et al., 1982b), transformants
were assayed for the presence of H-2^d gene hybrid products by radioimmunoassay. The hybrid antigens are expressed on the cell surface of recipient cells at levels comparable to those observed on H-2L^d, H-2D^d or H-2K^d transfectants. Screening of L cells with a panel of monoclonal antibodies mapped serological determinants to N and/or C1 as well as C2 domain of class I molecules and suggested that the polymorphic sites recognized by antibodies are encoded by single exons. Immuno-precipitation studies on some of the recombinant products demonstrated that hybrid antigens are ~45,000 dalton glycoproteins that associate with β2-microglobulin. Some transflectants were also tested in biological assays for their ability to act as targets for alloreactive cytotoxic T lymphocytes (Woodward et al., 1982) and after viral infection, as targets for virus-specific T killer cells (Örn et al., 1982; Forman et al., 1983). Our results indicate that most of the polymorphic determinants recognized by receptors of allogeneic and virus (vesicular stomatitis, lymphocytic choriomeningitis)-specific T cells restricted by L^d molecule reside in the N and/or C1 domain of the L^d molecule. Presently, we continue to characterize sites of T cell interactions in other hybrid antigens.

Using the same techniques, we are studying genes encoded in Qa-2,3 region of H-2 complex. One of these genes, 27.1 (Steinmetz et al., 1982), believed to be a pseudogene or a gene encoding secreted class I product, was recombined in vitro with parts of H2K^d, H-2L^d and H-2D^d genes. Serological, biochemical and biological analyses of the hybrids expressed on L cell transflectants demonstrated that N and/or C1 domains of 27.1 carry determinants recognized by Qa-2,3 region-specific cytotoxic T cells and polymorphic epitopes which crossreact with anti-K^d monoclonal antibodies. Gene 27.1, like other class I genes, appears to have its own functional promoter and ribosome binding site. We are planning to test whether it is also subject to γ-interferon-induced transcriptional regulation. These approaches will be extended to other genes in Qa-2,3 region in order to study their coding capacity, expression and, possibly, function.

These experiments are carried out in collaboration with Dr. M. McMillan (USC Medical School, Los Angeles, California) and Dr. J. Forman (University of Texas Health Science Center, Dallas, Texas).

References:

51. STRUCTURAL ANALYSIS OF THE E^B GENE

Investigators: Joan A. Kobori, Lloyd M. Smith, Eric Strauss, Karyl Minard

The class II genes of the mouse major histocompatibility complex encode four biochemically-defined polypeptides: A^B, A^Q^a, E^B and E^Q^a. These Ia (I region-associated) polypeptides are believed to play a role in cell-cell interactions among B cells, T cells and macrophages. They also appear to affect immune responsiveness in that the ability of certain animals to respond to a particular antigen is dependent on Ia antigens. The I-A antigen is a heterodimer of A^a and A^b polypeptides and the I-E antigen is a heterodimer of E^a and E^b polypeptides. The α chains are approximately 33,000 daltons in molecular weight and the β chains are approximately 29,000 daltons in molecular weight. Classical genetic mapping indicates that the genes for A^α and A^β and E^α and E^β are encoded in the I-A region and the E^a gene is encoded in the I-E region.

Steinmetz et al. (1982) isolated overlapping cosmid clones of BALB/c mouse spanning the I region. From these clones they were able to identify the E^α, E^β, A^β and A^α genes. We have defined the three major exons of the E^β gene by hybridization to a human DC-B cDNA probe (Long...
et al., 1982). This probe hybridizes most strongly to the mouse $A_B$ gene which shows significant hybridization homology to $E_B$. Unlike the other class II genes analyzed, the $E_B$ gene has a very large (~4.5 kilobases) intron separating the two major protein coding domains. Complete DNA sequence analysis of this gene is being pursued. This information will permit directed site-specific mutagenesis and modification of the $E_B$ gene in vitro to analyze the structural elements of the gene necessary for expression and biologic function in vivo. In addition, this information will be important in our analyses of I region recombinants (see Abstract No. 52). By analyzing restriction site polymorphisms characteristic of a given parental haplotype and by analyzing several I region recombinant strains, we can conclusively say that the $E_B$ gene maps into both the I-A and I-E subregions.

References:

52. ANALYSIS OF I REGION RECOMBINANTS OF THE MOUSE MAJOR HISTOCOMPATIBILITY COMPLEX

Investigators: Joan A. Kobori, Astar Winoto, Janet H. McNicholas*

Using Southern DNA hybridization techniques, restriction enzyme site polymorphisms have been used to correlate the molecular maps of the murine major histocompatibility complex I region with the genetic map of recombinant mouse strains. These data indicate that the molecular distance separating the I-A and I-E subregions is limited to 3.4 kilobases of DNA at the 3' end of the $E_B$ gene (Steinmetz et al., 1982). In accordance with recombinational analysis by serological techniques, this region should encode the I-B and I-J subregions (Figure 1). This amount of DNA is unlikely to encode even one complete murine gene in addition to the paradox that it lies within the $E_B$ gene.

To further study this problem, we isolated the $E_B$ gene region in question from four parental strains (B10.02, B10.A, C57BL/10 and AWS) and six I region recombinant strains (B10.A(3R), B10.A(4R), B10.GD, B10.HT and B10.S(9R)) and cloned it into a lambda phage vector. By direct restriction enzyme site mapping we have confirmed the location of the recombination region previously identified and have narrowed it down to approximately 1.5 kilobases of DNA. This region encompasses part of the intron between the first (81) and second (82) major exons and part of the second major exon (82) of the $E_B$ gene. Thus we can conclude that the I-B and I-J subregions as mapped by the immunogeneticist is contained within 1.5 kilobases of DNA and is within the $E_B$ gene. Clearly, a serologically detected difference between B10.A(3R) and B10.A(5R) has been observed in the I-J subregion; however, so far, at the molecular level they are identical. There are two major implications of this work: 1) The I-B and I-J subregions may not be encoded between the I-A and I-E subregions, and 2) a hotspot for recombination has been observed, suggesting that previous genetic mapping based on random recombinational frequencies should be reevaluated.

Reference:

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53. WHERE IS THE I-J STRUCTURAL GENE?

Investigators: Mitchell Kronenberg, Ellen B. Kraig, Judith A. Kapp*, Carl W. Pierce*, Tomio Tada**

The I region of the murine major histocompatibility (MHC) has been divided by analysis of recombinant inbred mice into several subregions arranged in the order I-A, I-J and I-E. The I-J subregion has been reported to encode polypeptides expressed by suppressor T cells. While numerous
alloantisera and monoclonal antibodies specific for I-J gene products have been defined, little is known about the biochemistry of I-J-encoded polypeptides. Interest in the I-J subregion and its gene products has been heightened by the finding that I-J-encoded polypeptides are associated with or are part of the antigen-binding polypeptides secreted by suppressor T cells. Thus, a biochemical characterization of I-J molecules may help to define the T cell antigen-binding receptor.

Recently, we have obtained cosmid clones derived from BALB/c spleen DNA containing all of the DNA sequences between the I-A and I-E subregions where I-J should be encoded (Steinmetz et al., 1982). Hybridization of these sequences to poly(A)+ RNA prepared from 13 I-J-positive suppressor T-cell hybridomas did not reveal the presence of any transcript. We estimate that our detection limit was 3-4 copies per cell.

The hybridization probes were derived from sperm DNA, and it is possible that the genomic organization of the I-J subregion is different in suppressor T cells than it is in sperm. However, we have analyzed the DNA from five different suppressor T-cell hybridomas using Southern blots, and in no case was any DNA rearrangement in the vicinity of the I-J subregion observed. The T-cell hybridomas used were derived by fusion of normal immune T cells from various inbred mouse strains to an AKR strain T-cell hybridoma. In four of the T hybridomas, we observed all of the hybridizing restriction fragments expected for both the normal parental DNA and the T lymphoma DNA. However, a fifth hybridoma retains hybridizing restriction fragments characteristic of the AKR T lymphoma, but appears to have lost the DNA fragments derived from the normal immune T cell parent. Subsequent analysis has provided evidence that the entire set of MHC genes derived from the normal T cell parent has been deleted in this hybridoma. Since this cell expressed the I-J allele of the normal T cell parent, we suspect that the I-J gene may in fact be located outside of the MHC.

Our analysis has proven that the genetic map position for the I-J gene is incorrect. It is possible that I-J is a serological artifact that has somehow spawned a vast literature. Alternatively, we have postulated that I-J may not itself be encoded in the MHC, but may be involved in the recognition of MHC-encoded molecules (Kronenberg et al., 1983). Currently, we are attempting to isolate I-J cDNA clones using specific monoclonal antibodies.

References:

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54. GENES OF THE MAJOR HISTOCOMPATIBILITY COMPLEX OF THE RAT

Investigators: Austin G. Diamond, Jonathan C. Howard, Astar Winoto

The major histocompatibility complex (MHC) is that part of the mammalian genome that codes for cell-surface molecules involved in lymphocyte interactions during immune responses.

This laboratory has previously identified the genes that code for the major serologically-identified loci in the mouse MHC and has started to define their precise positions within the complex (Goodenow et al., 1982; Steinmetz et al., 1982; Winoto et al., 1983). We have used the same approach to study the MHC of the rat which, although an important species in laboratory experiments, has been far less extensively characterized.

We have constructed cosmid libraries from two rat strains, PVG (which carries the MHC [RT1] haplotype c) and the MHC congenic PVG RT1B. These have been screened using as probes DNA isolated from the mouse libraries associated with the various genes of the MHC. To date a total of 63 cosmid clones have been isolated containing sequences homologous to various portions of the mouse MHC, probably including all previously identified genes of the I region. Currently, these clones are being mapped with restriction endonucleases to permit the establishment of overlapping fragments, and thereby their linear order in the genome.
Using transfection and monoclonal antibodies, we expect to be able to identify those genes that code for products of the known rat MHC loci. This will allow the study of the function of these molecules in immune responses. In addition, the comparison of the genetic organization of the two rat haplotypes together with those of the mouse should help elucidate the evolution of the MHC both within and between these closely-related species.

References:

55. CLONING OF PLATELET-DERIVED GROWTH FACTOR
Investigators: Astar Winoto, Paul Tempst, Richard K. Barth, Lance Fors

Platelet-derived growth factor (PDGF) is a heat-stable (100°C), cationic protein. It is stored in the α-granules of platelets and is released into the serum during blood clotting. It is a mitogen for fibroblasts, smooth muscle cells and glial cells, and is a major growth factor protein in the serum. Active PDGF consists of two chains with molecular weight ranging from 28,000 to 35,000 daltons. PDGF promotes phosphorylation of tyrosine at the PDGF receptor, an activity similar to that found for the products of several oncogenes. The amino terminal sequences of PDGF have been determined (Antoniades and Hunkapiller, 1983), and a search through the computer data base of Doolittle (Doolittle et al., 1983) reveals a striking homology with the transforming gene (v-sis) from the woolly monkey sarcoma virus. Since v-sis has only one cellular counterpart (c-sis), the homology finding suggests very strongly that PDGF is c-sis.

We have set out to clone and to characterize the cDNA and genomic clones of PDGF. The cloning and characterization of the PDGF gene will facilitate the later study of PDGF biological activity, especially in relationship to the transformed phenotype of a number of spontaneous human sarcomas. We have constructed a cDNA library from a human osteosarcoma cell line which secretes a PDGF-like protein. We also are currently constructing cDNA libraries from the osteosarcoma cell line using Okayama and Berg approach and λgt10 approach to clone the 4.2 kb mRNA transcript encoding the c-sis. Using the oligonucleotides corresponding to the PDGF chain 1 and 2 as well as v-sis as probes, we will characterize PDGF at both the cDNA and genomic levels.

References:

56. CLONING OF TRANSFORMING GROWTH FACTOR
Investigators: Astar Winoto, Paul Tempst

Transforming growth factors (TGFs) are polypeptides that are secreted by several transformed cell lines. TGFα is 7000 daltons and it competes with EGF for the EGF receptor. TGFβ is 13,000 daltons. When both are added to normal kidney cells, TGFα and TGFβ will confer transformed phenotype to the cells. The entire amino acid sequence of the TGFα has been determined in our laboratory (Marquardt et al., 1983). We are cloning the TGFα with the hope of understanding the relationship between growth factors and tumors.

A λgt10 cDNA library has been constructed from the rat ST-108 cell line which is secreting TGF. The library was screened using an oligonucleotide probe synthesized corresponding to the amino acid sequences of TGFα. A positive clone has been identified with 800 bp insert. We are currently sequencing the clone to confirm its identity.

Reference:

57. MOLECULAR CHARACTERIZATION OF THE GENES FOR MYELIN BASIC PROTEIN
Investigators: Arthur Roach, Kevin Boylan*, Stanley Prusiner*

Mammalian myelins contain a family of basic proteins called the myelin basic proteins (MBPs). An autoimmune response against at least one of these MBPs is found in patients with multiple sclerosis, and may be responsible for the degeneration of myelin and appearance of "plaques" of
demyelination in the white matter of afflicted individuals' brains.

The previously determined amino acid sequence of the small MBP from rat (Dunkley and Carnegie, 1974) allowed us to use a synthetic oligonucleotide probe to isolate clones encoding MBP from a cDNA library made from brain RNA from 18-day-old rats. Using these clones we intend to determine the basis for heterogeneity amongst rat MBPs. We will also attempt to characterize the human MBP gene and study its possible involvement in disease. Further, the existence of strains of mice genetically deficient in MBP provides a series of interesting questions which may be partially answered by studies at the molecular level.

Reference:

*Department of Neurology, School of Medicine, University of California, San Francisco.

58. DEVELOPMENT OF MICROSCALE PROTEIN PURIFICATION METHODS

Investigators: Michael W. Hunkapiller, Eva H. Lujan

Protein microsequencing technology developed in this laboratory over the past several years (see Biology 1982, No. 60) is now in routine use in the Biology Microchemical Facility at Caltech and is available commercially to other laboratories. The sensitivity of the sequencing methodology is now limited by the methods used to prepare small quantities of proteins for sequence analysis. We have developed a preparative technique based on continuous elution SDS-polyacrylamide gel electrophoresis that is suitable for sequencing studies. This method employs a disc gel electrophoresis tube that holds the gel through which the protein samples are electrophoresed and separated. Elution buffer is slowly pumped past the end of the gel to sweep the protein exiting the gel into fraction collector tubes. Aliquots of the fraction contents are analyzed on microslab SDS gels using ultra-sensitive silver staining. Fractions containing desired proteins are pooled, electrodialyzed, and lyophilized. The method is suitable for quantities of protein ranging from 0.5 to 20 μg and allows separation of proteins differing in molecular weight by as little as 500 daltons.

59. CHEMICAL SYNTHESIS OF PEPTIDES

Investigators: Stephen B. H. Kent, Suzanna J. Horvath

The chemical synthesis of peptides has recently become a tool of increasing importance in biological research in a wide variety of areas such as immunology, neuroendocrinology, cell biology and tumor biology. Perhaps the most important new application involves the synthesis of peptides derived from DNA sequence information. These peptides are used to generate antisera for use as laboratory reagents in identification and isolation of encoded proteins, and to follow their post-synthetic processing (Walter et al., 1981; Green et al., 1981; Lerner, 1982). The ability to chemically synthesize small proteins is valuable for the study of structure-function relationships in a number of classes of proteins with important biological activities.

We are using an improved version of the stepwise solid phase method to chemically synthesize peptides. Recent breakthroughs in understanding the fundamental nature of the solid phase (Kent, 1980) have led to the development of an optimized new set of chemistry, resin supports, and quantitative monitoring procedures, and to the elimination of several classes of by-products. The resulting high efficiencies (>99.8% per cycle) and speed (synthetic cycles typically 90 minutes per amino acid), enable rapid, routine synthesis of peptides of 50 or more residues. An automated synthesizer expressly designed to take advantage of this improved methodology is being developed.

Areas under study using chemical peptide synthesis include: use of domain-specific anti-peptide antisera to investigate the class I histocompatibility antigens (with Professor Minnie McMillan, USC); use of monospecific anti-peptide antisera to investigate the distribution and function of protein growth factors such as colony stimulating factors and tumor growth factors (with Dr. Michael Hunkapiller); investigation of structure-function relationships in the family of insulin-related growth factors, by total chemical synthesis. It is hoped that this type of approach can be combined with the study of receptor protein structure and function to elucidate mechanisms of cellular regulation and differentiation. We are
also planning to study the relationship of specific neural activity to local peptide structure, including separation of multiple activities residing in a single molecule, in the family of neuropeptide hormones giving rise to the characteristic behavioral array associated with egg laying in Aplysia (with Professor Felix Strumwasser).

References:

60. AUTOMATED CHEMICAL SYNTHESIS OF DEOXYOLIGONUCLEOTIDES

Investigators: Suzanna J. Horvath, Joseph R. Firca, Carol L. Graham

Chemically synthesized deoxyoligonucleotides with defined sequence have played an important part in much of the basic and applied research of molecular biologists at Caltech.

We have been routinely synthesizing DNA molecules up to 40 bases in length with excellent yield and high speed. Our improved, fully automated DNA synthesizer (Hunkapiller et al., 1983) utilizes a modified, highly efficient solid-phase phosphoramidite chemistry. By optimization of the machine performance, the cycle time for the addition of one nucleotide decreased from 1 hour to 10 minutes while the coupling yield slightly increased (>95% per cycle). When using protein amino acid sequence information, a set of heterogeneous DNA probes containing related but not identical sequences must be synthesized in order to obtain a sequence which covers all the coding possibilities for a given stretch of polypeptide. The unique design of the machine allows us to synthesize these mixed probes with single runs. The synthesizer is able to automatically insert multiple bases in equimolar ratio in any position of the DNA chain. We have been able to synthesize as many as 64 deoxyoligonucleotide sequences simultaneously. The mixed probes synthesized on the machine have been successfully employed in a variety of different experiments, e.g., genes were identified in isolated cosmids which genetically mapped to the immune response region from the major histocompatibility complex of the mouse (Steinmetz et al., 1982). Our DNA probes also have been used for mapping genes from gene families, for cloning of rare messages, and in site-directed mutagenesis.

References:

61. MICROISOLATION OF PROTEINS AND PEPTIDES FOR AUTOMATIC SEQUENCING

Investigators: Paul Tempst, Michael W. Hunkapiller

The development of the highly sensitive gas phase protein sequenator made possible the structure analysis of proteins that are only available in microgram quantities. Amino acid sequences can be backtranslated to the DNA sequences from which they could have been generated. Subsequently, synthetic oligonucleotides can be synthesized to screen cDNA libraries.

Final purification of proteins for sequencing is done by HPLC, gel electrophoresis or a combination of the two. Highly sensitive detection after electrophoresis is carried out by silver staining or fluorography of 14C-methylated proteins. These methods have been used in the isolation of human bone morphogenetic protein and a bacterial hydrolase.

In order to screen oligo(dT)-primed cDNA libraries for mRNAs containing large 3' untranslated regions, as well as for specific priming of reverse transcription, it is important to have oligonucleotide probes located as 3'-most as possible. To obtain amino acid sequences from the C-terminal region of a long protein, we are studying the chemical and enzymatic fragmentation of small quantities of proteins and limited cleavages by restricted activity of common endoproteases. The fragments can be subjected to microsequencing after separation by HPLC. This also generates partial sequence data from N-terminal blocked proteins. The extended sequence
data facilitates the search for minimal ambiguity oligonucleotide probes.

Using volatile solvents and short (C-3 and C-4) alkyleilane-bonded large pore size silicas, peptides and proteins in a size range from 5 amino acids up to more than 100,000 daltons can be separated in a single experiment with recoveries higher than 90% (Tempst, Hunkapiller and Hood, manuscript in preparation). Unresolved mixtures can be further separated on CN columns. These peptide isolation methods have been used in the study of the glucocorticoid receptor.

**PUBLICATIONS**


Assistant Professor: Elliot M. Meyerowitz
Visiting Associate: Ellsworth H. Grell
Gosney Research Fellow: Leslie S. Leutwiler
Graduate Students: Madeline A. Crosby, Thomas E. Crowley, Mark D. Garfinkel, Christopher H. Martin, Robert E. Pruitt
Research Staff: Bonnie K. Pruitt, Anne M. Villeneuve
Laboratory Staff: Robin K. Wilson

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Summary: Our laboratory continues to investigate the molecular mechanisms of organismal development, using the laboratory fly Drosophila melanogaster, and the flowering plant Arabidopsis thaliana, as our experimental material. Most of our Drosophila work is the study of one chromosomal locus, the 68C puff, found on the polytene chromosomes of salivary glands in the third instar stage of larval development. This puff is a site of coordinate transcription of three different RNA species during the third instar. This region is not transcribed at a high level in earlier or in later stages of development, and DNA sequences identical to those transcribed from the 68C puff in salivary glands are not detectably transcribed in tissues other than salivary glands at any time in development. These facts lead us to ask: what mechanisms cause the tissue- and developmental stage-specific expression of these DNA sequences? We know little about the signals in the salivary gland that start transcription of the three 68C RNAs; a considerable amount of evidence demonstrates that the steroid hormone ecdysterone, or 20-hydroxyecdysone, directly causes the near cessation of puff activity that occurs at the end of the third larval instar. In the past year we have confirmed our previous hypothesis that each of the 68C RNAs is a messenger RNA translated in the salivary gland to one of the glue polypeptides used by the larva to affix itself, and the pupa that it will become, to its substrate for the duration of the pupal period (Crowley et al., 1983). We also have completed the determination of the complete DNA sequence of 6,751 base pairs of puff DNA, including the entire sequence of all three RNA-coding regions (Garfinkel et al., 1983). This sequence shows that the three coordinately expressed genes share few sequence elements that might act as identical control sequences, and also shows that the three glue proteins coded at the puff are evolutionarily related to each other. The differences between the proteins appear to be due to two evolutionary processes: base substitution in the DNA, which at
times leads to amino acid differences between the proteins, and modular evolution, in which a large block of protein-coding DNA sequences appears in a particular member of the gene family, but not in others. The following abstracts detail other aspects of this year's progress, in particular a demonstration that ecdysterone works very rapidly in repressing accumulation of newly synthesized 68C puff RNA in isolated salivary glands, and the successful beginning of what will be a series of experiments in which fragments of the 68C gene cluster are cloned and amplified in bacterial cells, the 68C DNA purified and mutagenized in vitro, then reintroduced into the Drosophila genome. This reintroduced DNA can then be assayed for its transcription in the salivary glands, to determine the effects of the induced mutations on gene expression.

A second set of experiments that uses Drosophila is an investigation of the development of the fly eye, which develops from an undifferentiated epithelium to a highly ordered collection of 22 different cell types by processes that depend on communication between cells, and which require that individual cells know where they are in relation to cells of other types. We are currently analyzing several mutations that prevent particular aspects of this process of pattern formation.

A third series of experiments reintroduces the experimental use of higher plants to the Biology Division, after an absence of some years. We are initiating basic studies in the molecular genetics of a green plant not used before for this type of investigation, in the hope of developing a model system for the study of developmental processes unique to, or best studied in, plants. The plant is Arabidopsis thaliana, a member of the mustard family whose short generation time, low chromosome number and small size make it a convenient organism for laboratory studies. The past year's work has added to these previously known properties of Arabidopsis a property useful for recombinant DNA work: an extremely small nuclear genome, containing few if any dispersed middle repetitive DNA sequences. In addition, several new lines of study have been initiated with this organism, as described below.

The final abstracts detail technical advances in our abilities to work with cloned DNA fragments and to detect specific RNA sequences in Drosophila tissues; the application of these new techniques will bring us closer to our goal of understanding how plants and animals develop.

References:

62. REGULATION OF DROSOPHILA GLUE PROTEIN GENES INVOLVES THE ACTION OF 20-OH ECDYSONE
Investigator: Thomas E. Crowley

It is thought that puffs on polytene chromosomes result from a high rate of transcription of the DNA included in the puffed region. In Drosophila melanogaster salivary glands, the steroid hormone 20-OH ecdysone controls the puffing of many chromosomal loci including position 68C on the third chromosome. This locus is puffed during the third larval instar when glue proteins are being synthesized, but regresses at the end of this stage as a result of a direct interaction with ecdysone.

Previous results from our laboratory indicate that the glue protein mRNAs transcribed within the 68C puff are coordinately regulated such that they first appear in salivary glands during the third larval instar, and then disappear at the end of this instar. To determine if ecdysone is responsible for the rapid disappearance of these mRNAs, we measured the accumulation of newly synthesized RNAs in salivary glands cultured in vitro with the hormone. Dissected salivary glands were incubated in media containing the steroid at concentrations ranging from $10^{-17}$ M to $10^{-5}$ M and then pulse-labeled with $^{3}$H-adenosine for 15 min. The labeled RNA was subsequently purified and hybridized to an excess of filter-bound DNA homologous with each of the three 68C RNAs. By washing away unhybridized RNA and then quantitating the numbers of cpm on each filter, we determined what percentage of newly synthesized salivary gland mRNA corresponded to each of the 68C RNAs. Increasing the ecdysone concentration from $10^{-17}$ M to $10^{-5}$ M caused a tenfold reduction in the short-term accumulation of the sgs-3 mRNA and a twofold
reduction in accumulation of the sgs-7 and sgs-8 mRNAs. Since the labeling period was only 15 min in these experiments, we believe that the hormone reduces the rate of transcription or processing of the precursor RNAs in the nucleus, though until similar experiments with 3H-uridine are completed we cannot exclude the possibility that part of the incorporation observed is due to poly(A) addition in nucleus or cytoplasm.

To find the minimal time necessary for the hormone effect, dissected salivary glands were cultured in the presence of 10^{-5} M ecdysone for times ranging from 6 to 83 min before pulse-labeling. After the hormone treatment, RNA was labeled, purified and hybridized to an excess of 68C ONA as described above. A 6-min incubation with the steroid caused a 15% reduction in the amount of label incorporated into the sgs-3 mRNA, while a 20-min ecdysone treatment reduced incorporation into all three RNAs to its minimal level. The regression of the 68C puff was also analyzed in salivary glands cultured with 10^{-5} M ecdysone. A 2-hour incubation with the hormone was required to reduce the puff to its minimal size. The steroid appears to be modulating the expression of the 68C glue protein genes by a direct interaction since the effect is complete within only 20 min. It seems unlikely that 20 min would be long enough for regulatory proteins encoded at another locus to be synthesized and to interact with the 68C locus. Also, our results indicate that the size of a chromosomal puff may not be directly related to the rate of transcription of the DNA within the puff.

63. DETERMINATION OF SEQUENCES NECESSARY FOR REGULATED EXPRESSION OF THE Sgs-3 GENE

Investigator: Madeline A. Crosby

The expression of Drosophila salivary gland secretion proteins is specific to one tissue, the salivary glands, and to one stage of development, the third instar larva. The Sgs-J gene is the largest of three sgs protein genes located at 68C. This gene cluster has been completely sequenced and regions of transcription accurately mapped (Garfinkel et al., 1983). I am interested in defining the regions of the Sgs-3 gene that are necessary for its regulated expression.

It is now possible to make specific modifications within regions of a cloned gene in vitro and, by techniques developed by Rubin and Spradling (1982), insert the modified gene into the fly genome. The method of Rubin and Spradling uses a transposable element of Drosophila, the P element, as a mediator of transformation. The use of this technique allows the effect of known mutational lesions introduced in vitro to be assayed in the whole organism.

Before applying this method to investigations of the control of the Sgs-3 gene, I repeated the experiments of Rubin and Spradling using the rosy gene. I microinjected a mixture of two plasmids, one containing 8.1 kb of the wild-type rosy gene region inserted into a P element and the other containing an intact P element, into 47 ry^{+2} embryos. Ten of the animals survived the injection procedure; of these, three survived to adulthood and were fertile. All three of the injected animals that survived to adulthood exhibited a ry^{+} phenotype, indicating that the injected DNA was functioning in somatic cells. Integration of the injected DNA into germline cells also occurred, since one of the three animals produced offspring which showed the ry^{+} phenotype.

P-factor transformation of the Sgs-3 gene is slightly more complicated, since Sgs-3 itself does not confer a selectable phenotype. I constructed a plasmid which contains within the P element both the wild-type alcohol dehydrogenase gene (Goldberg, 1983) and 6 kb of the 68C region, including the Oregon-R wild-type Sgs-3 gene. I microinjected this plasmid plus a plasmid containing an intact P element into embryos which were Adh^{fn23} and carried the Hikone-R size variant of the Sgs-3 RNA. Transformants were detected by screening for ethanol-resistant animals among the adult F_1. Of 233 injected embryos, 16 survived to adulthood and were fertile; of these 16, one produced ethanol-resistant offspring.

Offspring of the resistant F_1 animals were dissected at late third larval instar and the salivary glands removed. The remainder of each larva was squashed and subjected to a redox indicator spot test to detect ADH activity (Grell
et al., 1968). The RNA from the salivary glands of sibling animals that scored positive or negative for ADH activity was run on an agarose-formaldehyde gel, blotted to nitrocellulose, and probed with labeled sequences homologous to Sgs-3 (Figure 1).

![Figure 1](image)

**Figure 1**

Sgs-3 gene introduced by P-factor transformation is transcribed in vivo. Total RNA from larval salivary glands was run on an agarose-formaldehyde gel (one pair of glands per lane), blotted to nitrocellulose, and probed with labeled sequences homologous to Sgs-3. Lanes 1 and 2 show the Sgs-3 RNA size variants present in the Oregon-R and Hikone-R strains, respectively. The Hikone-R strain carries a small duplication which includes Sgs-3 and thus shows a more intense autoradiographic signal. Lanes 3-5 show the Sgs-3 RNAs from offspring of an F1 ethanol-resistant animal backcrossed to the Adn n23 Sgs-3 stock. The animal in lane 3 was positive for ADH activity and also expresses the injected Oregon-R Sgs-3 gene; its two siblings in lanes 4 and 5 were negative for ADH activity and express only the endogenous Hikone-R Sgs-3 genes.

All animals that showed ADH activity also expressed the injected Oregon-R Sgs-3 genes as well as the endogenous Hikone-R size variant. Those animals that were negative for ADH activity showed only the activity of the endogenous genes. Thus, a 6 kb fragment from 68C, which includes 2.3 kb 5' of the Sgs-3 gene and 2.5 kb 3', is sufficient for expression of Sgs-3 in salivary glands.

In subsequent experiments, RNA blots of salivary glands from early third instar larvae and from pre-pupae were prepared and probed. No Sgs-3 mRNA was detected at these stages, neither from the endogenous genes nor from the injected genes. The carcasses of late third instar larvae (everything except the salivary glands) were also tested. The Sgs-3 message was not detected in any other tissue. Thus, the injected gene appears to be functioning only at the appropriate time and in the appropriate tissue.

**References:**


**64. SEQUENCES NECESSARY AND SUFFICIENT FOR THE IN VIVO EXPRESSION OF THE Sgs-7 AND Sgs-8 GENES**

Investigator: Mark D. Garfinkel

The salivary gland polytene chromosome puff 68C contains closely linked genes for three abundantly expressed salivary gland-specific mRNAs (Meyerowitz and Hogness, 1982). The genes are related to each other by gene duplication and divergence (Garfinkel et al., 1983), code for components of the salivary glue (Crowley et al., 1983), and are directly repressed by the steroid hormone 20-hydroxyecdysone (see Abstract No. 62).

The organization and coordinate regulation of this gene cluster raises several important questions: Is there a functional basis for the clustering of the three genes? Is each gene regulated by a discrete set of cis-acting regulatory sequences, or is there a single "global" regulatory site for all three genes?

Cloned DNA, manipulated in vitro, may be reintroduced into the fly genome by the methods described by Rubin and Spradling (1982). Cloned DNAs containing the P transposable element can be microinjected into Drosophila embryos, and the P element will transpose into germ cell precursor chromosomal DNA. Once integrated, the transposed DNA is stably inherited in future fly generations. "Defective" P elements can be prepared in vitro which carry genes of interest as well as another gene which confers a scorable or selectable phenotype upon flies possessing the integrated P element. The "defective" P elements transpose into germ cell chromosomes if co-injected with a fully functional "helper" P element (Rubin and Spradling, 1982). Two selectable markers we have used code for the enzymes xanthine dehydrogenase.
(XDH) and alcohol dehydrogenase (ADH) which give a
scorable eye color or resistance to ethanol,
respectively, when used to transform strains of the
appropriate enzyme-deficient genotype (see Abstract
No. 63).

Two of the three genes at 68C, Sgs-7 and Sgs-8,
are transcribed in opposite directions with their
5' ends 0.48 kb apart and their 3' ends 1.3 kb
apart. Just beyond the 3' ends are found a pair of
highly conserved 285 base pair repeat elements
which, like the genes themselves, are inverted
relative to each other. The two genes share
extensive nucleotide sequence similarity in the
first 100 base pairs upstream of each 5' end. The
third gene at 68B, Sgs-3, has a different sequence
adjacent to its 5' RNA terminus. A test of which
of these DNA sequences are required for the tissue­
specific expression, and steroid repression, of
Sgs-7 and Sgs-8 is in progress.

In order to distinguish transcripts of newly
introduced gene copies from those of the resident
genes, a restriction fragment was inverted so that
two fusion genes derived from Sgs-7 and Sgs-8 were
created. One fusion gene should give rise to a
novel larger RNA translatable to form sgs-8 pro­
tein, while the other fusion gene should give a
smaller RNA with no protein reading frame. This
inversion-fusion gene pair has been transferred to
a P-ADH plasmid and microinjected into ADH­
deficient embryos. Among a small sample of
injected animals, progeny which had acquired
resistance to ethanol have not yet been obtained.
Further injections and screening, and vector
constructions to make use of XDH are in progress.

References:
Crowley, T. E., Bond, M. W. and Meyerowitz, E. M.
Garfinkel, M. D., Pruitt, R. E. and Meyerowitz, E.
28, 165-176.
218, 348-353.

65. COMPARATIVE STUDIES OF THE 68C REGION IN
DROSOPHILA SPECIES RELATED TO D. MELANOGASTER
Investigators: Christopher H. Martin,
Elliot M. Meyerowitz

A region homologous to the 68C glue puff
sequences of D. melanogaster has been isolated from
each of four other species of the melanogaster
species subgroup. Our aim is to compare the glue
gene coding regions and surrounding areas at the
restriction site, RNA transcript, and sequence
levels. It is hoped that these studies will reveal
conserved regions within the 68C region which may
be directly related to the regulation of the glue
genes. Such studies should also lead to insights
into the evolution of this gene cluster, in
particular into the modular relation between the
three proteins coded by it (Garfinkel et al., 1983).

Characterization at the restriction site level
has been completed. Comparison of the restriction
maps of the cloned regions of the five species has
shown that there is little conservation of restric­
tion sites in the glue protein coding regions and
to the right (towards the centromere) of this
region. However, a large region to the left of the
glue gene cluster is highly conserved in its
restriction site pattern.

Currently, studies are being carried out on the
gene organization of the glue genes in these other
species. This includes the determination of the
number of genes coding for glue proteins, their
location, and orientation of transcription. Preliminary
results indicate that the gene cluster
may be evolving by duplications, insertion of
transposable elements, and inversions of glue
genes. These findings may have implications on the
relationship of the organization of the cluster to
regulation of the glue genes within it, and may
point out some of the processes of evolution that
act to change the DNA sequences in and around
regulated structural genes.

Reference:
Garfinkel, M. D., Pruitt, R. E. and Meyerowitz, E.

66. DEB MUTAGENESIS IN THE 68A-D REGION
Investigators: Madeline A. Crosby,
Christopher H. Martin

On the basis of genetic tests, Shukla and
Auerbach (1980) claimed that the mutagen diepoxy­
butane (DEB) induces a large percentage of "small"
deletions. They estimated that two-thirds of DEB­
induced mutations are deletions large enough to
uncover a viable visible mutation as well as a
lethal mutation.
In an effort to recover small deletions in the 68C region, we conducted a screen for DEB-induced lethals over a deficiency extending from 68A3 to 68B1. Ten lethals were recovered from 2950 chromosomes tested; this frequency is comparable to that observed by Shukla and Auerbach. By deficiency mapping it was determined that seven of these lethals map to 68A, a region in which we have 47 ethyl methane sulfonate (EMS) and ethyl nitrosourea (ENU)-induced lethals that define at least 14 complementation groups. None of the DEB-induced lethals appears to uncover more than one complementation group. By comparison, three of the 22 EMS-induced lethals uncover two or more complementation groups.

Although the frequency of small deletions among the total number of EMS-induced mutations is relatively low (14% in this case), EMS is a more efficient mutagen than DEB. The three deletion mutants described above were recovered from a screen of 2227 EMS-mutagenized chromosomes, in contrast to zero such mutants recovered from 2950 DEB-mutagenized chromosomes.

Reference:

67. SEARCHING FOR THE FUNCTION OF A NOVEL REPEATED DNA FAMILY

Investigator: Mark D. Garfinkel

The glue protein genes Sgs-7 and Sgs-8 at polytene chromosome bands 68C3-5 are surrounded by two inverted copies of a highly conserved 285 base pair repeat element. Three more copies of the repeat element are found in a 6,000 base pair segment of DNA near the polytene band 68C10 proximal to the 68C3-5 copies (Biology 1981, No. 85). I wish to determine the function of this repetitive DNA family. Are the elements themselves transcribed? Are they responsible for salivary gland-specific abundant expression of adjacent genes? Are they associated with additional glue protein genes or pseudogenes? Several lines of evidence argue against a role for the repeat elements in regulating expression of known glue protein genes. First, at 68C3-5 there are three abundantly expressed salivary glue protein genes (Sgs-3, Sgs-7 and Sgs-8), only two of which are adjacent to members of the repeat family. Second, the 68C3-5 region is prominently puffed during the third larval instar period when the genes are active, while there is no observed puffing near 68C10. Third, when radioactive cloned DNA containing the three 68C-proximal repeat elements is used in salivary gland RNA gel blot hybridization experiments, no homologous RNA is detected. When a more sensitive inverse experiment is done—cloned DNA from 68C10-11 is restriction enzyme-digested, subjected to agarose gel electrophoresis adjacent to restricted cloned DNA from 68C3-5 and blotted to nitrocellulose filters—hybridization of single-stranded 32P-labeled DNA complementary to third instar larval salivary gland poly(A)+ RNA can be detected. Restriction fragments containing two of the three proximal repeat copies retain about fiftyfold less radioactivity than fragments containing each of the three glue genes at 68C3-5. The 32P-labeled cDNA bound to the 68C10 fragments remained after stringent (2 mM Na+, 50°C) washing of the filter, which suggests that well-matched duplexes form between the cloned DNA and the 32P-labeled cDNA. Since 68C3-5 fragments containing only the repeat elements failed to hybridize 32P-cDNA, I conclude that the hybridization observed to 68C10-11 DNA is due to a relatively rare component of the salivary gland 32P-cDNA which is homologous to unique sequences adjacent to the repeat elements.

68. CHARACTERIZATION OF MUTATIONS AFFECTING DEVELOPMENT OF THE DROSOPHILA EYE

Investigators: Anne M. Villeneuve, Elliot M. Meyerowitz

The adult Drosophila eye is composed of a regular hexagonal array of 700 to 800 ommatidia. Each ommatidium is itself a regular array of several different specialized cell types. Two major processes, pattern formation and differentiation, are involved in the development of this highly structured tissue (Ready et al., 1976). As a first step in understanding which of these two processes is being affected by a variety of mutations that cause abnormal eye development in Drosophila, somatic recombination spots containing tissues mutant for each of these loci are induced
in otherwise wild-type eyes. (For dominant mutations, the spots are wild-type in mutant backgrounds.) Each spot is simultaneously marked with an autonomous eye color mutation and an eye development mutation, and the phenotype of the spot and its surrounding tissue is studied (Meyerowitz and Kankel, 1978). If the developmental mutation is autonomous—that is, presumably acting at the level of differentiation—the border of the mutant phenotype and that of the autonomous eye color marker will coincide. If the mutation is working at the level of pattern formation, a variety of processes, such as communication between neighboring cells of the action of a diffusible substance through the tissue, may be affected. The boundaries of the mutant phenotype might extend outside of the color spot, or the wild-type phenotype may extend into the color spot, depending upon the specific process or interaction involved. Mutations of this type are called non-autonomous.

Of the 21 mutations studied, only two appear to be non-autonomous. The first of these, Irregular facets (If), produces eyes with misshapen facets in disarray. In If mosaics, there often appear to be areas of wild-type ommatidia extending well beyond the boundary of the color spot marking recombinant cells. This effect is variable, and nonspecific with respect to location of the spot within the eye.

The second putative non-autonomous mutation is Microcephalus (Mc), a mutation with a variable phenotype in which the eye is greatly reduced in size, and at times entirely absent. Somatic recombination in Mc flies fails to produce any recovery of the wild-type phenotype, even in large color spots.

Histology is currently being done on adult eyes in order to study the phenomena involved in these mutations in greater detail. Information obtained through this histological study will determine the direction of future analysis of the mutations, and will aid in the elucidation of the mechanisms by which they disrupt pattern formation.

References:

69. CHARACTERIZATION OF THE GENOME OF ARABIDOPSIS THALIANA

Investigator: Leslie S. Leutwiler

Plant genomes exhibit a wide variation in nuclear DNA content. Generally plants contain higher proportions of repetitive DNA and methylated nucleotides than animals. We chose to do molecular biology studies on Arabidopsis thaliana, a member of the mustard family, because nuclear staining techniques showed that it had one of the smallest genomes of the angiosperms (Bennett and Smith, 1976). We have characterized the genome by measuring the reassociation kinetics of DNA extracted from whole plants in order to determine the size of the nuclear genome and to characterize the DNA with respect to repetitive sequence content.

Total DNA was extracted from whole plants and mixed with two labeled internal tracers: $^{32}$P-nick translated DNA from a $\lambda$ clone containing a chloroplast DNA insert was used as an internal standard to monitor reassociation conditions; $^{3}$H-nick translated whole plant DNA was used to follow the reassociation of the plant DNA. The reassociation of the $^{3}$H-labeled DNA gave a two-component curve. The complexity of each component was then calculated from their reaction rates using the known complexity of E. coli DNA and its rate of reassociation under the same conditions. The kinetics of reassociation of the rapidly reassociating component were very close to the value for the $^{32}$P-labeled internal tracer; this component is therefore presumed to be chloroplast DNA. It makes up 25% of the total DNA and has a complexity of 125 kb and copy number of slightly more than 400 in a diploid cell. The unique component, which composed 50% of the total DNA, reassOCIated with a rate indicating a complexity of 50,000 kb. Of the remaining 25%, about half is due to a highly repetitive component that reassOCIated at very low Cot values and 10% may be a middle repetitive component that cannot be resolved by such kinetic measurements.

As mentioned above, plants contain a relatively large amount of 5-methylcytosine. Angiosperms contain an average of slightly more than 5% of their nucleotides as 5-Me-C (Shapiro, 1975). We
used Drosophila embryo DNA containing no 5-Me-C as control samples to analyze by HPLC the 5-Me-C content of Arabidopsis. The results show that Arabidopsis contains slightly more than 1% of its bases as 5-Me-C. This is the lowest value reported for any of the angiosperms (Shapiro, 1975). These studies have shown that the Arabidopsis genome is extremely small—no larger than 75,000 kb (0.08 pg)—that it lacks any detectable middle repetitive DNA, and that it contains only a very small amount of methylated nucleotides.

References:

70. ANALYSIS OF RANDOM A. THALIANA CLONES

Investigator: Robert E. Pruitt

We have extended our analysis of randomly chosen clones of Arabidopsis DNA in phage λ vectors to include 24 clones (see Biology 1982, No. 79). Fourteen of these clones have been examined by forward and reverse genome blots, while only reverse genome blots have been performed on the remaining 10. Of these clones, 20 appear to contain DNA that is unique in the Arabidopsis genome; two appear to be moderately repetitive and two appear to be highly repetitive. The two highly repetitive clones contain five EcoRI fragments of identical sizes. By restriction enzyme mapping, each of these clones was determined to contain 1.5 tandem repeats of a 9.8 kb repeating unit. By Northern blot analysis, one of these clones was shown to hybridize to the nuclear-encoded ribosomal RNAs of A. thaliana. One of the two moderately repetitive clones appears to be of chloroplast origin based on genome blot hybridization and hybridization to partially purified chloroplast DNA. The other clone has not been examined in detail.

The clones identified as unique are being used as probes to construct a genetic map of A. thaliana based on restriction fragment length polymorphisms (RFLPs). Different wild-type strains of Arabidopsis have different restriction maps for a given segment of DNA. These differences can be detected by probing genome blot filters of the different strains using the unique probes. In genetic crosses between strains, these differences can be followed in the same manner as classical genetic markers and linkages between RFLPs determined. To date we have examined the genomes of five wild-type strains with seven unique probes. Three of these clones showed polymorphism between at least two strains. This indicates that it should be possible to isolate the 50 or so polymorphic clones that will be required to give a fairly complete map of the Arabidopsis genome.

The ratio of single-copy repetitive and chloroplast clones found so far is generally consistent with the kinetic analysis described in Abstract No. 69; the absence of dispersed repetitive elements in the 20 single-copy clones implies that the Arabidopsis genome has little dispersed repetitive DNA.

71. USE OF Ti PLASMIDS AS TRANSPON MUTAGENS

Investigator: Robert E. Pruitt

The plant pathogen Agrobacterium tumefaciens causes the neoplastic disease crown gall by a novel mechanism known as genetic colonization. The bacterium carries a tumor-inducing (Ti) plasmid and during infection a small portion of this plasmid is transferred to the plant cell nucleus where it is stably integrated into the host genome (Chilton et al., 1977). The site of the integration event appears to be relatively nonspecific (Thomashow et al., 1980). Integration of this transferred DNA (T-DNA) into a gene presumably results in loss of function of that gene. Experiments are currently in progress to use Ti plasmids to induce mutations in Arabidopsis tissue culture cells. By infecting cells which are heterozygous for mutations that result in biochemically selectable phenotypes, we hope to be able to select cells in which the T-DNA has integrated into the remaining wild-type copy of the selectable marker. The plasmid sequences may then be used as a probe to obtain molecular clones of the gene of interest from recombinant DNA libraries. During the last year we have worked out the required tissue culture techniques for
72. CONSTRUCTION OF DIRECT SELECTION VECTORS

Investigator: Robert E. Pruitt

In the construction of recombinant DNA clones, one major problem is the selection of recombinant vs. vector-only molecules. To overcome this, it would be desirable to design a selection system that discriminates against vector-only clones, resulting in the death of the host cells containing them. Towards this end, vectors have been constructed based on the ß-complementing lacZ system used in the pUC series of vectors. The new vectors are similar to the pUC (Rodier et al., 1981) vectors but contain a kanamycin resistance gene in place of the ampicillin resistance gene and also tend to replicate at higher copy number than the pUC vectors. DOA-1 is a conventional plasmid vector while DOA-2 incorporates the ß cohesive end fragment (cos) and should function as a cosmid vector. In order to take full advantage of these vectors, a new E. coli host strain (BOZO 2.7) was also developed. The genotype of BOZO 2.7 is \( \mu^c \mu^f \) srl[Tn10, Tet\(^R\)] recA56 lacZAM15. We currently use these vectors to select for inserted clones by plating on kanamycin-containing MacConkey/lactose plates and picking Kan\(^R\), lac+ colonies. A more general method involves the use of a compound that is converted into a toxic material by the action of ß-galactosidase, resulting in the death of non-recombinant clones. We have synthesized and analytically tested chloramphenicol-ß-D-galactoside (death-gal) for this purpose and have found it capable of selectively killing lacZ* E. coli cells. Large-scale synthesis and testing is planned but has not been carried out to date. Additional vectors are under construction which incorporate an SP6 phage promoter downstream of the lac region and oriented in the opposite direction. These vectors will be useful for the production of single-stranded RNA probes from inserted DNA which can be used to improve the sensitivity of tissue in situ hybridizations being carried out in this laboratory (see Abstract No. 73).

References:

73. IN SITU HYBRIDIZATION OF DNA TO RNA IN DROSOPHILA TISSUE SECTIONS

Investigator: Anne H. Villeneuve

The technique of in situ hybridization of DNA probes to RNA in tissue sections is a valuable tool for the study of spatial and temporal expression of genes during development. The goal of this project has been to optimize the technique so that it can be used for a variety of purposes in our research. The method currently in use in our laboratory is a synthesis of modifications of methods described by a number of investigators (M. Akam, unpublished observations; Angerer and Angerer, 1981; Brahic and Haase, 1978; Godard and Jones, 1979).

Preliminary hybridization experiments were done using as probes tritium-labeled cDNA clones from messages which are abundant in and specific to salivary gland tissue in third instar Drosophila larvae. These probes were hybridized in situ to cellular RNA in 10-micron-thick larval serial sections, and autoradiography was done in order to visualize the hybridization signal. After optimization of various steps in the protocol, I have been able to obtain consistently strong specific signal with low background using tritium-labeled probes. I have also successfully used 35S-labeled DNA probes in hybridization experiments. Since the strength of the signal is roughly proportional to the specific activity of the probe, the higher specific activities possible using 35S rather than tritium should allow the localization of messages which are not abundant.

Ultimately, we would like to increase the sensitivity of the method in order to detect rare messages. This is possible only with the use of single-stranded DNA or RNA probes. We are currently constructing plasmids containing a viral
promoter that will be used to produce large quantities of single-stranded RNA for use as probes in RNA-RNA in situ hybridization. It should be possible to obtain high signal-to-noise ratios using this method, since RNA-RNA hybrids are extremely stable, non-hybridized label can be removed by ribonuclease treatment, and single-stranded probes are not removed from the in situ hybridization reaction by annealing to their complement in solution, as are double-stranded probes.

References:

Professor: Herschel K. Mitchell
Senior Research Fellow: Nancy S. Petersen
Research Fellow: Donald J. Silvert
Research Staff: Carmen M. Parseghian, Joan Roach

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Summary: Our major interests of many years have been directed toward studies on the regulation of gene expression as related to programs of differentiation. To this end we make use of electron microscopy to follow details of morphogenesis and protein and mRNA synthesis to define the molecular components that are involved in the structures and processes which constitute morphogenesis. Most recently we have made extensive use of differentiation of cell hairs in Drosophila wings as a unique model system. The special usefulness of the system is derived from the fact that about 30,000 wing cells construct cell hairs synchronously in the absence of cell division. This occurs during pupal stages when a single wing can provide sufficient material to follow the rapid changes that occur in both transcriptional and translational activities. We make extensive use also of a variety of existing mutants of Drosophila that affect morphology as well as numerous phenocopies that mimic the mutants. The latter provide information on the times at which specific morphological events occur. We continue to study the heat shock and the stress protection phenomena in relation to phenocopy production as well as with respect to the phenomenon itself.

74. HAIR MORPHOGENESIS IN THE DROSOPHILA MUTANT mwh

Investigators: Herschel K. Mitchell, Joan Roach, Nancy S. Petersen

Each wing cell in the wild-type Drosophila differentiates to produce a single hair under normal circumstances. In contrast, the mutant multiple wing hair (mwh) produces from three to six hairs per cell. They may be similar in size or quite variable but it appears that the total surface area remains about the same. We have shown by electron microscopy of thin sections that the hairs of mwh are extruded suddenly in the 33-34 hr pupal stage, just as has been demonstrated for the wild type (Mitchell et al., 1983). In the mutant, multiple hairs appear at this time rather than 4-5 hr later as in the multihair phenocopy (Mitchell and Petersen, 1982).

A comparison of protein synthesis patterns in wings of OreR and mwh during the period of hair extrusion showed several polymorphisms that might
be related to the mutant character. However, all but one of these differences disappeared in a mutant stock which carried the wild allele of mwh only in females as a translocation of the tip of chromosome 3L (61 to 62AB) to the X chromosome. In this stock, females are wild and males are mwh in phenotype. Stocks that are mwh produce an abundant protein with a molecular weight of about 25,000 as do + stocks but the isoelectric point is about one half pH unit higher in the mutant. Further studies are anticipated at the cloning level.

References:

75. PROTEIN COMPONENTS OF WING HAIRS IN DROSOPHILA
Investigators: Herschel K. Mitchell, Nancy S. Petersen

We have shown earlier that Drosophila wings provide a unique system for studies of the regulation of differentiation (Mitchell and Petersen, 1981, 1983a). That is, rapid changes occur in mRNA and protein synthesis during the time when about 30,000 wing cells (non-dividing) produce cell hairs in synchrony. We have now identified at least 10 of the most abundant proteins that are associated in some fashion with this multistep process. Hairs can be stripped off of wing surfaces at various stages of differentiation and analyzed for protein content. A major component with a molecular weight of about 60,000 daltons is obtained when hairs first appear at 33 hr. Three of the smaller components described earlier (Mitchell et al., 1983b) appear in the hair preparations within the next 2 hr. These components are candidates for structural materials that are involved in the formation of cuticulin.

References:

76. ISOLATION OF GENOMIC CLONES CODING FOR MAJOR MESSENGER RNAs EXPRESSED IN WING DEVELOPMENT
Investigators: Nancy S. Petersen, Herschel K. Mitchell

The isolation of clones coding for the major messages involved in wing development is a first step in looking for the regulatory mechanisms that regulate their sequential expression. Messenger RNAs have been isolated from five stages of wing development where all of the major messenger RNAs are different. cDNAs to these RNAs have been used to screen the Drosophila library made by Joyce Lauer at Caltech. Clones have been isolated that encode major messages for each of these stages. These will be identified further by in situ hybridization to polytene chromosomes to determine their chromosomal locations and they will be used to select mRNA for translation to determine which of the proteins in the wing program they encode. Some of the clones will be selected for further study—particularly one that encodes a major protein that is only synthesized when actin is not being synthesized. We also will study those whose chromosomal location and time of expression suggest that they may be the genes responsible for specific mutant or phenocopy defects.

77. STABILITY OF HEAT SHOCK PROTEIN
Investigators: Nancy S. Petersen, Herschel K. Mitchell

Decay of hsp 70 is correlated with increased heat sensitivity of D. melanogaster larvae. A brief heat treatment at 35°C greatly improves the ability of Drosophila larvae to survive a higher temperature treatment (30 min at 40°C). The effect of the 35°C treatment on survival following a second heat shock depends on the length of time between the 35°C and 40°C heat shocks (Petersen and Mitchell, 1983). A delay of 0.5 to 1 hr at 25°C between heat shocks increases survival. After the first 2 hr the effect of the 35°C treatment on survival gradually decays over a period of about 30 hr. Larvae that have been injected with 35S-methionine incorporate label into heat shock protein for about 2 hr after the 35°C treatment. All of the labeled heat shock proteins except hsp 70 are stable over a 24 hr period. The hsp 70
(as seen on a one-dimensional SDS acrylamide gel) decays to less than 10% of the original label while hsp 60 remains completely stable. This suggests that hsp 70 may be essential for the effect of the 35°C treatment on survival following heat shock.

Reference:

78. HEAT SHOCK MUTANTS

Investigators: Nancy S. Petersen, Carmen M. Parseghian, Herschel K. Mitchell

Heating animals to temperatures that induce heat shock protein synthesis along with normal protein synthesis can protect larvae, pupae, adults and cell lines from the normally lethal effects of a subsequent high-temperature treatment. Circumstantial evidence strongly indicates that the survival protection and the protection against phenocopies are due to the expression of some or all of the heat shock genes. During the current year we have started a mutant hunt using a heat shock survival screen. We hope to use mutants with altered recovery from heat shock to a) determine which heat shock gene products may be involved in enhancing survival and preventing developmental defects following heat shock, b) study regulation of heat shock gene expression, and c) explore the molecular basis for the phenocopy prevention and survival protection.

The screen that we are using to select mutants involves comparing survival of larvae that are heated under two different conditions (A, 40.8°C for 30 min; B, 35°C for 30 min followed by 40.8°C for 30 min) with survival of larvae that remain at 25°C. Normally, larvae die if heated 30 min at 40.8°C (condition A) and about 50% survive if they are heated to 35°C before the 40.8°C treatment. In order to look for mutants, males are mutagenized and individual stocks of flies with mutagenized chromosomes are grown up and tested. We have tested 1850 individual stocks for X chromosome mutations affecting heat shock survival or normal development. We have found 80 that do not survive following the 35-40.8°C heat shock and 32 that show developmental abnormalities following this treatment. We also are looking for third chromosome mutations that affect survival following heat shock.

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Summary: We are interested in the development of T lymphocytes. These include both the cells that directly lyse foreign or virus-infected cells, and the cells that positively and negatively regulate the magnitude of almost all immune responses. Unlike B lymphocytes, which are morphologically similar, T lymphocytes do not make immunoglobulins. An important issue, then, is precisely which genes need to be expressed in order to make a T cell functionally competent.

The types of gene products that are required for function can be classified into four expression groups. First, there are antigen-specific receptors that are known to be expressed constitutively on the T-cell surface. Many laboratories have tried to identify these receptors, but their structures are still largely unknown. Second, there are the polypeptides that control T-cell proliferation, which are only induced in T cells after the binding of antigen. These polypeptides include the T cell-specific growth hormone, IL-2, and the IL-2 receptor. Third, after antigen-triggered proliferation has generated a clone of effector T cells, there are the various molecules directly used in immunological attack mechanisms, either to lyse target cells (e.g., "lymphotoxins") or to stimulate or suppress other lymphocytes' responses. For helper T cells, IL-2 could also be regarded as an effector-molecule in this category. Finally, the fourth type of product to consider is the hypothetical class of mutagenic enzymes that may contribute to T-cell receptor diversity, probably in very early stages of T-cell development. One provocative candidate is terminal deoxynucleotidyl transferase (TdT). Our interest is focused on the second and fourth types of molecules, to ask how programmed mutagenesis may be regulated in T-cell differentiation and when growth control first becomes linked to antigen recognition.

Killer and helper activities are carried out by distinct types of T cells, but what all T cells have in common is that their precursors migrate to the thymus and proliferate there before emerging as immunologically competent lymphocytes. Among the proliferating thymocytes are, therefore, developing T cells in a stage somewhere between pluripotent hematopoietic stem cells and mature T cells. These clearly make an interesting population to study.

We would like to ask to what extent the dividing thymocytes (lymphoblasts) are already functionally competent; whether they are already committed to helper or killer lineages; and whether they actively mutate their DNA in a physiologically productive way.

The red herring in this type of inquiry has traditionally been the majority population of thymocytes, which is composed of functionally inert, postmitotic cells that linger in the thymus for 3-4 days and then die. To avoid confusing the properties of these cells with those of the lymphoblasts, we have spent several years developing cell fractionation techniques to isolate the lymphoblasts physically. This has enabled us to identify one biosynthetic marker specific for lymphoblasts, the TL glycoprotein. Our separation technology has also allowed us to analyze the heterogeneity within the lymphoblast population, an essential step towards understanding when sublineages of T cells diverge. We have begun to assay the functional reactivities of different types of lymphoblasts and are now embarking on a dissection of their reactivity at the molecular level.
their ability to produce the T cell growth factor, interleukin-2 (IL-2). IL-2 plays a crucial role in the proliferation of activated T lymphocytes and its production identifies the producing cell as a "helper" T cell.

The elaboration of IL-2 activity from thymocytes in response to mitogenic stimulation requires co-stimulation with the phorbol ester TPA. Under these conditions the amount of IL-2 produced is increased ten to twentyfold (Rothenberg et al., 1983; Caplan and Rothenberg, in preparation). We have localized the IL-2-producing thymocytes to a subpopulation of "mature" thymocytes defined by their lack of expression of receptors for the lectin peanut agglutinin. Within these peanut agglutinin-negative thymocytes, there are at least three phenotypically distinct populations of IL-2 producers. We have identified both Lyt2-positive and Lyt2-negative IL-2 producers in the thymus (Caplan and Rothenberg, in preparation). This is in contrast to recent reports that only Lyt2-negative thymocytes are capable of producing IL-2. In addition, we have established that a proportion of the large, proliferating, thymic blast cells can produce IL-2 (Caplan and Rothenberg, in preparation). These cells may represent a self-renewing population of mature IL-2 producers or a stem cell population of helper T cell precursors already capable of producing IL-2.

The extension of this work will be directed towards establishing the molecular basis for the observations described above. Towards this end we hope to obtain a cDNA clone for the IL-2 gene and to begin an analysis of the events involved in activating T cells to produce IL-2.

Reference:

80. AN ASSAY FOR DETERMINING THE FREQUENCY OF THYMOCYTES THAT ARE CAPABLE OF PRODUCING INTERLEUKIN-2 (IL-2)

Investigators: Thomas J. Novak, Barry I. Caplan, Ellen Rothenberg

Recent work in this lab on the immunocompetence of murine thymocytes has identified several phenotypically distinct populations that can produce IL-2 under appropriate conditions. These sub-populations can be identified by several physical and phenotypic criteria: size, Lyt antigen expression and binding to peanut agglutinin. However, we lack any estimate of the frequency of IL-2 producers within given subpopulations. Therefore, we are developing an assay for detecting IL-2 production by a single cell.

The assay utilizes a clonal murine cytotoxic T-cell line (MLT2.8.2) which has an absolute dependence on IL-2 for growth and therefore serves as indicator cells. These cells are grown in bulk culture in IL-2-containing medium until the growth factor is exhausted. The IL-2-depleted cells, which will die within 24 hr if IL-2 is not added, are allowed to form a confluent monolayer on the bottom of a 60 mm petri dish containing a protamine-coated polyester insert. This monolayer of indicator cells is then overlaid with a known number of polyclonally-stimulated thymocytes in a semi-solid matrix of 0.7% low melting temperature agarose. After 19 hr of incubation 5 µCi of 125I-UdR in 0.2 ml of phosphate-buffered saline is added directly onto the agarose and allowed to diffuse through to the indicator cells. Five hours later the agarose is removed and the indicator cells are fixed to the polyester insert. We hope to determine the frequency of IL-2 producers in the thymocyte population by placing the insert under film or coating it with photographic emulsion and exposing it for several days. Indicator cells that were directly under an IL-2 producer should have begun to replicate their DNA, and the incorporated label will give rise to spots on the film or emulsion. So far, we can establish consistent monolayers of indicator cells and obtain a clear autoradiographic distinction between those that have and have not received IL-2.

The advantage of this assay over a limiting dilution analysis is that it detects IL-2-producing cells within 24 hr of their removal from the thymus, not several days later. It will permit us to relate the activity of cells directly to their differentiation stage in the thymus and to measure the ability to secrete IL-2 separately from the ability to grow in culture.
81. PURIFICATION OF MOUSE INTERLEUKIN-2 (IL-2) AND AN ASSESSMENT OF ITS EFFECTS ON VARIOUS THYMOCYTE SUBPOPULATIONS

Investigators: Santosh Krishnan*, Janes P. Lugo, Ellen Rothenberg

We are interested in determining the effects of lymphokines such as IL-2 on various thymocyte subpopulations. Preliminary results have indicated that subcapsular thymocytes (large thymic lymphoblast cells) have the capability to respond to IL-2. Since the IL-2 used in these experiments was in a crude supernatant, the effect on these cells of mitogens other than IL-2 could not be assessed. Therefore we need to purify IL-2 to a significant level of homogeneity before more conclusive results can be obtained.

The discovery that IL-2 does not bind to the lectin Concanavalin A (Con A) (Clark-Lewis and Schrader, 1982) but does to the lectin Wheat Germ Agglutinin (WGA) shows that lectin chromatography is one way of purifying this protein. Using lectin chromatography and DEAE-sephacel chromatography, we have achieved a 35-fold enrichment of IL-2 from crude supernatants. Using high-pressure liquid chromatography, we expect significantly higher degrees of purification of IL-2.

The purification of this reagent will allow us to assess rigorously the kinetics of induction of IL-2 receptors on thymocytes and peripheral T cells and assist in providing interpretable results concerning the responsiveness of various thymocyte subpopulations to this hormone.

Reference:
*Undergraduate, California Institute of Technology.

82. TL GENE EXPRESSION IN SUBPOPULATIONS OF THE THYMIC ANTIGEN

Investigators: Rochelle D. Sailor, Catherine Kirschvinck, Douglas A. Fisher, Ellen Rothenberg

We are investigating the control of gene expression of differentiation antigens in subpopulations of thymocytes and their counterpart leukemic cell lines. As immature T cells differentiate in the thymus, they experience programmed changes in their expression of a variety of gene products. TL (thymus leukemia antigen) is one such gene product. TL is a class I hematopoietic differentiation antigen encoded on chromosome 17 in the mouse. Within the thymus, TL antigens are expressed only on cortical thymocytes. The majority of these cells, the small nonproliferating cells, express TL antigen on their surface but only actively synthesize a very small amount of TL. A minority of cortical cells, the large proliferating blast cells (10-15% of the population) actively synthesize most of the TL found in the thymus as measured by metabolic pulse labeling and quantitating TL by immunoprecipitation from de novo synthesis (Rothenberg, 1982). Looking at de novo synthesis rates of TL, it appears to be developmentally regulated at the translational or pretranslational level. The antigen has been characterized by using specific alloantisera and monoclonal antibodies recognizing different TL determinants for normal and leukemic cell populations. Thus by the appearance of TL message in large proliferating blast cells and not in their progeny or precursors, TL expression can be used as a marker for thymocyte differentiation.

Two genomic clones for TL antigen have been isolated from a λ phage genomic library of BALB/c DNA containing the H-2 gene locus and shown to produce TL antigens when transformed into mouse L cells (Goodenow et al., 1982). In collaboration with Douglas Fisher in Lee Hood's group, we are subcloning fragments of these phage inserts to generate DNA probes specific for TL genes, allowing rapid screening of TL gene expression and control. The goals of this work will be to provide some measurement of the transcription of TL sequences, to quantify the message in different subpopulations of thymocytes, and to determine the regulatory impact of outside factors on the transcription of the gene.

References:

83. INTRODUCTION AND EXPRESSION OF EXOGENOUS DNA IN MATURE T CELLS

Investigators: Thomas J. Novak, Ellen Rothenberg

Distinct subpopulations of murine thymocytes
have been identified by a variety of criteria. Thymocytes in the cortex, both the dividing lymphoblasts and the much larger number of nondividing cells, synthesize gene products that are not found in any mature lymphocytes. These include TdT and TL, two highly characteristic differentiation markers whose functions are unknown. We would like to be able to introduce such genes into lines of mature T cells to study their function and regulation.

To that end we are refining the selective conditions for the detection of T cells transformed with exogenous DNA. Two cloned lines of T cells are being studied: the helper-lineage T cell lymphoma, EL4.E1, and the IL-2-dependent cytotoxic T cell line, MTL.2.8.2. Calcium phosphate coprecipitation, protoplast fusion, and microinjection are being evaluated for their relative efficiency in introducing the DNA.

To detect successful DNA uptake and transcription, we are using a series of indicator plasmids constructed by Dr. Cornelia Gorman (NIH). These plasmids contain promoter and enhancer sequences from various sources and the structural gene for chloramphenicol acetyltransferase, an enzyme which is easily assayed and absent from normal mammalian cells. Stable transformants are scored by their ability to grow in the aminoglycoside drug G418 after transformation with the plasmid pNeo3. This plasmid, given to us by Dr. Barbara Wold, contains the pBR322 origin of replication and β-lactamase with the Tn5 kanamycin/neomycin resistance gene fused to an HSV thymidine kinase promoter and poly(A) addition site. Resistance to G418 provides a dominant selectable marker that should enable us to score transformation of normal primary lymphocytes. Unlike L cells, however, both T cell lines that we have tested are relatively refractory to killing by G418. In order to increase the efficiency of selection, putative transformants are cycled in and out of selection medium containing 1% glycerol as a permeabilizing agent. This has allowed us to isolate transformed colonies from both T cell lines. Their frequency is very low, however (10^-6 to 10^-7 per µg DNA), when standard calcium phosphate precipitation is used and only the HSV tk promoter is present.

**84. ISOLATION OF THE MOUSE INTERLEUKIN-2 (IL-2) RECEPTOR GENE**

Investigators: James P. Lugo, Ellen Rothenberg

We are attempting to clone the mouse IL-2 receptor gene. The strategy we anticipate using will be to introduce mouse DNA into an IL-2-dependent human T cell cloned line and then select for transformants capable of growing in mouse IL-2-containing medium. This approach bypasses the need to identify the IL-2 receptor protein altogether, and is based on the finding that IL-2-dependent human T cells are incapable of growing in media supplemented with mouse IL-2 (Robb et al., 1981). The recipient cell line we anticipate using in these studies is the MJ human retrovirus-infected T cell line, which has previously been shown to have a growth requirement for human IL-2 (Popovic et al., 1983). Our preliminary results confirm this observation and also show that mouse IL-2 does not satisfy this growth requirement. We are currently assessing the ability of these cells to take up and then transcribe exogenous DNA. Ultimately, we would like to use this gene as a probe for studying the molecular biological aspects of T-cell activation.

**References:**


**PUBLICATIONS**


Rothenberg, E. and Triglia, D. (1983) Clonal proliferation unlinked to terminal deoxynucleo-
structures in eukaryotic cells. This approach to the regulation of their expression and the relationship between gene structure and protein function. This work has further led us to try to understand the mechanisms that eukaryotic cells use to transduce temporal information acquired from their environment into intracellular signals. Thus, for a number of years we have worked with bacterial cells that are able to change their surface properties in order to accommodate to changing conditions. We have analyzed the genetic mechanisms involved in regulating cell-surface functions in bacteria. One of these is bacterial motility which is driven by bacterial flagella. Flagellar rotation responds to chemical substances in the environment and we therefore have been working on chemoreception and the mechanisms involved in the bacteria’s ability to detect gradients of attractants and repellents and to respond to stimuli. These cells use a series of specific receptors that bind attractants, e.g., amino acids or sugars, and are able to generate signals that are proportional to the concentration of ligand bound to the receptor as a function of time. We have cloned all of the genes involved in bacterial chemotaxis and in the formation of the bacterial flagella structure. We have been studying the regulation of their expression and the relationship between gene structure and protein function. This work has further led us to try to understand the mechanisms that eukaryotic cells use to transduce temporal information acquired from the environment into intracellular signals that change cellular metabolism. We have begun detailed studies on the protein transducin, which is necessary for visual adaptation. We are working on related proteins that appear to be involved in processing information from cell-surface receptors into intracellular signals. Finally, we are initiating work on the mechanisms involved in regulating the expression of genes that encode specific cell-surface structures in eukaryotic cells. This approach requires the development of methods for studying mammalian genetics and has led us to set up systems for cloning and mapping genes in the mouse.

**PUBLICATIONS**


**Summary:**
The Togaviruses are a family of animal viruses which have a nucleocapsid with icosahedral symmetry surrounded by a lipoprotein envelope. The envelope, although acquired by budding of the capsid through host cell membranes, contains only virus-encoded proteins. We are interested in the structure, replication and evolution of this family of viruses, with the focus of our work at present being on two genera in this family, the alphaviruses and the flaviviruses. Alphaviruses and flaviviruses are transmitted in nature by mosquitoes and have the ability to grow in the vector as well as in the vertebrate host. The vertebrate host range is broad: many of these viruses grow in a wide variety of birds and mammals and some of them probably include reptiles in their natural host range as well. This alternation in nature between invertebrate and vertebrate hosts has important implications for the evolution of these viruses.

The alphaviruses consist of about 20 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 have conducted some comparative studies with a number of pathogenic strains as well. We have now obtained the complete nucleotide sequence of the RNA genome of Sindbis virus, some 11,703 nucleotides, and from this we have deduced the details of the organization of the genome and the amino acid sequences of the encoded proteins. Comparative studies with other viruses have revealed that certain areas of the genome are highly conserved, whereas others change more rapidly. From these studies an evolutionary tree has been constructed. Sequence studies of temperature-sensitive mutants have revealed the sites of several mutations which lead to temperature sensitivity. Other studies have focused on the glycoproteins of Sindbis virus.

Work with the type flavivirus, yellow fever virus (we use the vaccine strain in our studies) is progressing rapidly. In addition, comparative studies with other flaviviruses have revealed details of the relationships of these viruses to one another.

**85. THE NUCLEOTIDE SEQUENCE OF THE SINDBIS VIRION GENOME**

**Investigators:** Ellen G. Strauss, Charles M. Rice, James H. Strauss

During the past year we have completed the nucleotide sequence of the genome of Sindbis virus and have obtained comparative sequences of regions of the Middelburg virus genome. Sindbis RNA contains 11,703 nucleotides, excluding the 5' methylated cap structure and the poly(A) tract of approximately 60 residues at the 3' terminus. The RNA contains a long reading frame for 2513 amino acids which is open except for a single opal termination codon (discussed in more detail below). This reading frame encodes the nonstructural (replicase) proteins and begins with an AUG initiation codon at nucleotides 60-62 and terminates with three in-phase stop codons (two amber, one ochre) at nucleotides 7599-7601, 7608-7610 and 7626-7628. The subgenomic message begins at nucleotide 7598, the last nucleotide of the final codon in the nonstructural polyprotein; there are only 48 nontranslated nucleotides before the AUG start codon for the structural proteins at nucleotides 7647-7649 of the genomic RNA (or nucleotides 50-52 of the subgenomic RNA). The structural proteins are encoded as a polyprotein of 1245 amino acids, which is processed by posttranslational processing to form two glycoproteins and a nucleocapsid protein. The coding region for the structural protein is followed by a 3' terminal nontranslated region of 322 nucleotides and the poly(A) tract.

The most interesting feature of the nonstructural protein region is the presence of an...
opal stop codon, UGA, at nucleotides 5748-5750. The polyprotein terminated by this stop codon is processed to form three proteins, probably all involved in RNA replication, which we refer to as nsP1, nsP2, nsP3 in order of translation. The sites of cleavage to produce these proteins have been determined only indirectly. We know from independent studies (see Abstract No. 92) that the opal codon is read through in vivo to produce a small amount of a fourth replicase component, nsP4.

To ensure that the termination codon did not represent a sequencing or cloning artifact, we have determined the sequence of a second alphavirus, Middelburg virus, in this region and found an opal codon at the same position. The corresponding nsP4 of Middelburg downstream of the termination codon contains 75% amino acid homology with that of Sindbis, suggesting that this read-through product is indeed translated in both cases and is essential for alphavirus replication.

Thus, alphaviruses, with a small and relatively simple genome, are capable of regulating the synthesis of their virus-encoded products at three different levels by two different mechanisms. Production of a subgenomic (265) RNA as the predominant message in infected cells insures that most of the virus-specific protein synthesis is devoted to production of the structural proteins. Translation of the genomic RNA up to the opal codon produces three nonstructural polypeptides in lesser amounts and read-through may produce a fourth poly­merase component in very small quantities for specific regulation of replication.

86. THE 5'-TERMINAL SEQUENCES OF THE GENOMIC RNAs OF SEVERAL ALPHAVIRUSES

Investigators: Jing-hsuing James Ou, Ellen G. Strauss, James H. Strauss

The 5'-terminal sequences of the genomic RNAs of several alphaviruses have been determined. The nucleotide sequences at the extreme 5' termini are not highly conserved among the alphaviruses, but a similar stem and loop structure, which begins at the 5' end and utilizes about the first 40 nucleotides, can be formed in each case. Downstream from this structure, beginning about 150 nucleotides from the 5' end, a conserved sequence of 51 nucleotides is found which can form two stable hairpin structures. Examination of the 5'-terminal and 3'-terminal sequences suggests that part of this conserved nucleotide sequence may be involved in cyclization of the RNA. A model is proposed for the function of the 5'-terminal sequences in RNA replication. In addition, sequence homologies among these RNAs strongly support the hypothesis that an AUG codon, which occurs at 60 to 80 nucleotides from the 5' end, depending on the virus, and which may or may not be the first AUG codon, is used for initiation of translation of the nonstructural proteins and allows a comparison of the deduced amino acid sequences in the NH₂-terminal regions.

87. SEQUENCE ANALYSIS OF TWO MUTANTS OF SINDBIS VIRUS DEFECTIVE IN THE INTRACELLULAR TRANSPORT OF THEIR GLYCOPROTEINS

Investigators: Carlos F. Arias-Ortiz, John R. Bell, Edith M. Lenches, Ellen G. Strauss, James H. Strauss

We have sequenced the complementary DNA corresponding to the genes encoding the viral glycoproteins of ts10 and ts23, mutants of Sindbis virus defective in the intracellular transport of their glycoproteins, and of revertants of these mutants. These studies have been augmented by direct amino acid sequencing of the amino-terminal regions of the glycoproteins of several virus strains. By comparing the deduced amino acid sequence with that of Sindbis HR virus, the parental strain of these mutants, and with the sequence of the revertants, we found ts23 to have a double mutation in glycoprotein E1, while ts10 was a single mutant in the same glycoprotein. In each case reversion to temperature insensitivity occurred by changes at the same site as the mutation, in two cases restoring the original amino acid and in the third case substituting arginine in place of lysine. The three mutations were far apart from each other in the protein, suggesting that the three-dimensional conformation is very important for the correct migration of the glycoproteins from the rough endoplasmic reticulum to the plasma membrane. The sequence data also reveal that a number of other changes have occurred in the various virus strains during mutagenesis or passage.
88. **Determination of the Mutant Defect in Sindbis Mutant ts20**

Investigators: Bjorn H. Lindqvist, Jerry D. DiSalvo

*ts20* is the only temperature-sensitive mutant of Sindbis virus which belongs to complementation group E. At the non-permissive temperature, the precursor to the glycoprotein E2 is not processed and no virus particles are produced. In order to localize the defect in this mutant, we have cloned a cDNA copy of the relevant region of the genomic RNA of *ts20* and of a revertant of it and sequenced the region encoding the structural proteins. Poly(A)-containing *ts20* 495 RNA was copied by reverse transcriptase using a plasmid vector containing a poly(T) tail as the primer. Second-strand synthesis was performed with *E. coli* polymerase I in the presence of RNase H and *E. coli* ligase. The double-stranded product was digested with Hind III and cyclized with ligase. Several clones were selected after transformation of *E. coli* bacteria and the cloned DNA was sequenced by chemical methods. The E2 protein contained a change from histidine (in the parental virus) to leucine (in *ts20*) at residue 291 which returned to histidine in the revertant.

89. **Ross River Virus 26S RNA: Complete Nucleotide Sequence and Deduced Sequence of the Encoded Structural Proteins**

Investigators: Lynn Dalgarno*, Charles M. Rice, James H. Strauss

Ross River virus (RRV), an alphavirus first isolated in Australia, is capable of causing periodic outbreaks of human disease, characterized by polyarthritic symptoms. Strains of RRV that differ in pathogenicity have been isolated in nature, and wild strains can be altered in pathogenicity by passage in tissue culture. The purpose of this study was to examine at the molecular level the evolutionary relationships between RRV and other alphaviruses with different geographical distributions, such as Sindbis virus and Semliki Forest virus (SFV), and also to begin collecting sequence data that should eventually allow the identification of changes in the RRV genome that cause the observed differences in pathogenicity between strains. Thus far we have determined the nucleotide sequence of the subgenomic 26S RNA of RRV that encodes the virus structural proteins. The RNA sequence was determined without the use of molecular cloning by chemical sequence analysis of end-labeled Hae III-digested cDNA fragments. These sequences were translated by computer into the three possible reading frames. The correct coding phase was identified by (1) a lack of termination codons, and (2) homology with the corresponding Sindbis virus and SFV structural protein sequences. By comparison with the known sequences of the 26S RNA, we could unambiguously order the RRV Hae III cDNA fragments. The overlap regions were then determined by sequencing appropriate double-stranded cDNA restriction fragments. The complete sequence reveals that the genomic organization of the RRV structural protein genes is identical to that of Sindbis virus and SFV. However, the 3'-untranslated region of RRV, 524 nucleotides, is more than 200 nucleotides longer than the corresponding region in Sindbis or SFV. Examination of structural protein sequences of these viruses revealed that RRV is much more closely related to SFV (75% homology between the structural proteins) than to Sindbis virus (47% homology). In addition, during the sequence analysis of RRV, a variant RNA population was detected that contained a 21-nucleotide deletion that encoded seven amino acids near the NH₂ terminus of the virion glycoprotein E2. This variant could be plaque-purified from the original RRV stock, and differed phenotypically from the parental strain by producing smaller plaques.

*Australian National University, Canberra.*

90. **Evolution of Alphaviruses**

Investigator: John R. Bell

The alphaviruses consist of about 20 members which are defined on the basis of serological cross reactions. In order to understand the evolution of these viruses, we have obtained the N-terminal amino acid sequences of the two glycoproteins of eight different alphaviruses, in collaboration with Dr. Dennis Trent and Richard Kinney of the Centers for Disease Control in Fort Collins, Colorado. These sequences also allow for more extensive comparisons in this group of viruses, and include
regions of protein sequence other than the epitopes responsible for virus neutralization.

The eight different viruses are all closely related, showing sequence homologies from 32% to 83% depending upon the pair of viruses being compared. Examination of these data by computer analysis has allowed construction of an evolutionary tree and supports the hypothesis that all alphaviruses have evolved from a common ancestor.

Despite the sequence conservation found, relatively few amino acid positions were inviolate in that some changes occurred at almost all positions. Positions conserved in all or almost all viruses over the regions sequenced include all cysteine residues, certain proline and glycine residues, and many of the aromatics. Since cysteine, proline, and glycine residues are important for protein folding and three-dimensional structure, this suggests that considerable variation in the primary sequence of the polypeptide can be accommodated while the overall conformation of the protein (and its activity) is conserved.

Glycoprotein E1 is more highly conserved among the alphaviruses than glycoprotein E2. Along with other evidence from our laboratory and from others, this suggests that E2 is primarily responsible for the strain differences that arise during evolution.

91. CONSTRUCTION OF SINDBIS VIRUS DEFECTIVE INTERFERING RNAs IN VITRO

Investigators: Charles M. Rice, Henry V. Huang*

From comparative sequence analysis, it appears that at least three regions of the alphavirus 495 genomic RNA are important in the replication of the genome and transcription of the 3'-terminal subgenomic 265 RNA. Two of these regions, the 5' and 3' ends of the 495 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 replicational machinery) and are thought necessary for production of full-length (-) and (+) 495 RNA. A third region (called the junction region), adjacent to the start of 265 RNA, may encode the recognition site for initiation of 265 transcription from the 495 (-) 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 495 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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92. IDENTIFICATION OF SINDBIS NONSTRUCTURAL POLYPEPTIDES BY SPECIFIC IMMUNOPRECIPITATION

Investigators: Susana López-Charreton, John R. Bell

During replication of Sindbis virus, two species of viral messengers are translated: the subgenomic 265 mRNA which encodes the virus structural proteins, and the genomic 495 RNA, which specifies the nonstructural polypeptides as a long polyprotein precursor. Nonstructural proteins are made in small quantities early in the infection cycle when host protein synthesis is still continuing. For this reason, these polypeptides are difficult to find in the background of host proteins. Using the known RNA sequence and the deduced amino acid sequence, we constructed two synthetic peptides, one 12 amino acids long whose sequence is found near the amino terminus of the polyprotein (NsP-N corresponding to amino acids 230-240) and the other 12 amino acids long whose sequence is found at the carboxyl terminus (NsP-C corresponding to amino acids 2503-2513). Rabbits were immunized with these peptides alone or with the peptides conjugated to bovine serum albumin. Four virus-specific polypeptides were immunoprecipitated with the anti NsP-C antisera with apparent gel molecular weights of 250,000, 200,000, 150,000 and 72,000. The 72 K protein represents a readthrough product (see Abstract No. 85) and the longer moieties are precursors to it.
93. PURIFICATION AND ANALYSIS OF THE SMALL NON-STRUCTURAL GLYCOPROTEIN (E3) OF SINDBIS VIRUS

Investigators: Jeffrey T. Mayne, Charles M. Rice, Michael W. Hunkapiller, Ellen G. Strauss, James H. Strauss

A small glycoprotein (E3) was purified from the culture fluid of Sindbis virus infected primary chick embryo fibroblasts. Tryptic peptide mapping and pulse-chase studies verified that this protein was produced as a by-product of the cleavage of the precursor protein PE2 to produce the envelope glycoprotein E2. A 2600-fold enrichment was achieved via a purification scheme involving differential ethanol precipitation, gel filtration, ion exchange chromatography, and affinity chromatography using a lentil lectin column. Amino acid composition analysis, N-terminal microsequencing and labeling studies yielded information about the fine structure of E3 and its relationship to E2 and virion maturation. The N-terminal sequence of E3 is identical to that of PE2, including the result that 90% of the molecules appear to be blocked. The first 19 amino acids are uncharged and presumably serve as the signal sequence for the insertion of PE2 into the membrane of the endoplasmic reticulum, but this sequence is unusual in that it is not immediately cleaved from PE2 and is glycosylated at the asparagine at position 14. The two C-terminal residues of E3, Lys-Arg, are removed during or shortly after cleavage from PE2. Labeling studies imply that although the PE2 + E2 + E3 cleavage is necessary for virion budding, these two events are not closely coupled. E3 is cleaved and released into the culture fluid under conditions where virions do not bud, and the kinetics of the appearance of E3 in the culture fluid and E2 in virions are quite dissimilar. The maturation of E3 is discussed as it relates to the processing of cellular membrane or secretory glycoproteins.

94. THE CARBOHYDRATE CHAINS OF SINDBIS GLYCOPROTEINS

Investigators: Jeffrey T. Mayne, John R. Bell

The tryptic glycopeptides of the Sindbis virus envelope glycoproteins E1 and E2 grown in BHK and chick cells were purified by gel filtration followed by high pressure liquid chromatography. Each of the purified glycoproteins was analyzed by N-terminal sequencing to identify from which of the potential glycosylation sites it was derived. The identity of the type of oligosaccharide chain attached to each glycopeptide was determined from gel filtration analysis of the pronase-digested glycopeptides, and the relative incorporation of radio-labeled galactose, mannose and glucosamine into each glycopeptide was used to confirm these determinations. We found that the glycosylation patterns for the two proteins were essentially identical in the two hosts. The E2 glycosylation sites at Asn196 and Asn398 contain exclusively complex type and simple type oligosaccharide chains, respectively. In E1, the glycosylation site at Asn135 contained only complex type chains, but the site at Asn245 contained a mixture of simple (15-20%) and complex (75-85%) type chains. These results have been compared to previous results and predictions made as to the relative importance of the different glycosylation sites to the function of the proteins.

95. ISOLATION AND CHARACTERIZATION OF HYBRIDOMAS SPECIFIC FOR SINDBIS VIRUS PROTEINS

Investigators: Jeffrey T. Mayne, Charles M. Rice

Hybridomas were selected by the fusion of NSI/1 myeloma cells with spleen cells from mice inoculated with either Triton X-100 treated Sindbis virions or with membrane preparations from cells infected with ts23, a Sindbis virus temperature-sensitive mutant that fails to cleave the precursor protein PE2 at the nonpermissive temperature. Ten stable hybridomas were obtained, seven specific for the envelope glycoprotein E1 and three specific for the capsid protein, and the immunoglobulin subclasses of these clones were determined. The seven E1-specific antibodies were divided into two classes, which reacted with different antigenic domains of E1, as determined by a binding competition assay. The two classes of antibodies differed in several tested properties. One class, referred to as the E1(a) class, included five of the clones and reacted with a protein preparation (E1 + E2 in Triton X-100) that the second class, called E1(b), did not. In addition, antibodies
from the E1(a) class precipitated intact virus to a much greater degree than antibodies from the E1(b). Two clones from the E1(a) class were able to inhibit viral infectivity, and one of these was able to precipitate E2 along with E1 in the Triton-treated virus preparations.

96. FLAVIVIRUS STRUCTURE AND REPLICATION

Investigators: Charles H. Rice, Edith M. Lenches, Rebecca Sheets*, Sean Eddy*

To complement the studies on flavivirus structural and nonstructural proteins already initiated in this lab (see Abstract No. 97), we have begun nucleotide sequence analysis of the vaccine strain of yellow fever virus (YFV). A cDNA library was constructed after reverse transcribing virion RNA into double stranded cDNA. A random primer was used for first strand synthesis so that the entire genome would be represented in the cDNA synthesized. Virus-specific clones with long inserts (2-7 kb) were selected for further analysis. Preliminary restriction mapping suggests that the YFV genome is at least 10.5 kb in length, and we are presently attempting to define the 5' and 3' ends by sequence analysis of the genomic RNA. Partial sequence and open reading frame analysis of the cloned DNA tentatively identified the cDNA strand corresponding to the YFV genomic RNA. Furthermore, beginning near the putative 5' end of the YFV genome a single open reading frame encodes the virion structural proteins (deduced by comparison with previously determined N-terminal sequence data) in the order N (nucleocapsid protein), M (matrix protein or small envelope glycoprotein), and G (the major virion glycoprotein). We are presently completing the nucleotide sequence of the YFV genome as well as initiating further studies to elucidate the genomic organization of the structural and nonstructural YFV proteins and define the molecular changes involved in attenuation of this important human pathogen.

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97. N-TERMINAL SEQUENCE ANALYSIS OF FLAVIVIRUS PROTEINS

Investigator: John R. Bell

The members of the flavivirus genus of the Togavirus family include many important human pathogens, including yellow fever virus and dengue virus. Originally, the flaviviruses were thought to be related to the alphaviruses on the basis of similar morphology and mode of transmission. Members of both genera are small enveloped viruses with an icosahedral nucleocapsid and one molecule of single stranded RNA of plus polarity as their genome. Both alphaviruses and flaviviruses replicate in their vertebrate hosts and in their invertebrate vectors. However, at the molecular level these two groups are quite dissimilar in the polypeptide composition of the virions and in their translation and replication strategies. Flaviviruses have one species of nucleocapsid protein of 13.5 kilodaltons, a glycosylated envelope protein of roughly 60 K and a small envelope polyprotein of approximately 8 K.

We have begun to characterize the structural proteins of several flaviviruses in collaboration with Dr. Dennis Trent and Richard Kinney from the Centers for Disease Control in Ft. Collins, Colorado. These include St. Louis encephalitis virus, dengue virus and yellow fever virus. To date the N-terminal amino acid sequence of the glycoprotein and of the nucleocapsid protein of all three viruses and of the small envelope polypeptide of two of the viruses has been determined. None of the sequences show any detectable homology with any of the alphavirus proteins, but the three flaviviruses are closely related to one another. They show clear amino acid sequence homology in all three proteins, and for the glycoproteins the sequence homology between any pair of flaviviruses is on the order of 50%. This is similar to the situation we have found among the alphavirus glycoproteins (see Abstract No. 90).

The amino acid sequences obtained for yellow fever virus have been used to align the structural proteins with the nucleotide sequence (see Abstract No. 96). In this way the details of the flavivirus replication strategy are becoming known, and it appears that at least the structural proteins are
produced by posttranslational cleavage of a polyprotein precursor.

98. PRIMARY STRUCTURE OF THE NEUTRALIZATION ANTIGEN OF SA11 SIMIAN ROTAVIRUS AS DEDUCED FROM cDNA SEQUENCES

Investigators: Carlos Arias-Ortiz, Susana López-Charreton, John R. Bell

Rotaviruses are agents of acute viral gastroenteritis in young children and in other young mammals and birds and as such are major human and veterinary pathogens. The viruses consist of 11 genomic segments of double stranded RNA encapsidated in two concentric protein shells. The major outer capsid polypeptide, VP7, is a glycoprotein encoded by RNA segment 9 and is thought to be the antigen eliciting neutralizing antibodies in the host.

To define the antigenic properties of VP7, we have obtained a cloned copy of segment 9 and determined the nucleotide sequence of the gene by chemical methods. The sequence of segment 9 is 1092 base pairs long and contains an open reading frame 978 nucleotides long which begins at an initiation codon at nucleotides 49-51 and terminates with the first of two in-phase termination codons at nucleotides 1025-1027. A second initiation codon in the same frame is present at nucleotides 136-138 and it is unclear at this point whether both initiation sites are used in vivo. That this indeed is the coding sequence for the outer glycoprotein was confirmed by partial amino acid sequencing of purified VP7 isolated from virions. The glycoprotein was blocked at its NH₂-terminus and thus could not be sequenced directly. However, a peptide generated by Staphylococcus aureus V8 protease digestion was isolated and found to contain a sequence corresponding to amino acids 221 to 237 as predicted from the nucleotide sequence, confirming the coding assignment and identifying the coding strand of segment 9.

PUBLICATIONS


Assistant Professor: Barbara J. Wold
Research Fellow: V. Craig Bond
Graduate Students: Elliot C. Altman, Paul K. Herman, Stuart K. Kim, Paul R. Mueller
Research Staff: Jessie Mei Huei Jong, Margaret S. Smith

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Summary: We are interested in the regulation, structure and physiology of animal cell-surface receptors. Thus far we have worked principally with the low density lipoprotein (LDL) receptor. These receptors provide the cell with cholesterol from extracellular sources by specifically binding LDL (the principal cholesterol transport molecule in serum) and subsequently internalizing it via receptor-mediated endocytosis. The receptor level is regulated over a wide range to provide appropriate quantities of cholesterol. When the supply of LDL is not sufficient, cells turn to an alternative source of cholesterol—de novo synthesis from acetate. In this pathway, the rate-limiting step is catalyzed by HMG CoA reductase (an early step in cholesterol biogenesis). We are interested in studying the basis of the delicate regulatory relationship between the receptor pathway and the de novo pathway, and we are interested in discovering how the receptors themselves work. Some of our efforts during the past year have been directed toward cloning DNA sequences encoding key gene products in cellular cholesterol metabolism.

We have continued our investigations of homologous recombination in animal cells. Our initial purpose was to develop a means by which LDL receptor mutants (or mutants of any other selectable gene) could be mapped with relative speed and ease. Studies with the herpes virus thymidine kinase (tk) gene have established the feasibility of recombination mapping in DNA transfer. The focus of these studies has now shifted to (1) attempting to develop a protocol by which heterologous donor tk DNA sequences will recombine with their chromosomal homologues; (2) attempting to understand the apparent differences between certain genes in the mouse major histocompatibility complex which seem to frequently undergo homologous recombination or gene conversion with donor DNA (Goodenow et al., 1983) and the tk model system in which such events appear to be exceedingly rare.

We also are interested in studying the basis of regulatory events in the cell utilizing DNA transfer techniques. The problems here include transcriptional regulation using the metallothionein and interferon genes as a model and specific DNA level rearrangement in which immunoglobulin heavy chain class switching is the event of interest.

Reference:

99. HOMOLOGOUS RECOMBINATION IN ANIMAL CELLS
Investigator: V. Craig Bond

I am utilizing the cloned herpes virus thymidine kinase (tk) gene to study homologous recombination in animal cells. Three major questions are addressed. (1) Can the cell perform the recombination events necessary to create an intact gene from the input gene fragments and stably express this intact gene? (2) What properties of the input DNA are important for these recombination events to occur efficiently? (3) Can these recombination events occur between input gene fragments and an endogenous gene?

The experimental approach is to transform cells with a combination of truncated tk genes that share 217 base pairs of homologous sequence. A single recombination event in the region of homology will regenerate a wild-type gene as one product. Recombination was assayed using two different procedures. One assays the production of stable transformants and therefore permits us to characterize the recombination events at the DNA level. The second assay was developed to take advantage of transient expression. This assay is very quick and allows large numbers of condition variations to be examined. Initial experiments showed neither of the cloned fragments could by itself transform tk- cells, but a mixture containing both plasmids
generates tk\(^+\) transformants. Linearizing both cloned fragments in the region of homology (in this case the Hind III sites) enhanced the frequency approximately tenfold over linearization at other sites, suggesting that initial homologous strand invasion may be facilitated by a cut in the region of shared sequence. Under these conditions we have observed \(>2 \times 10^2\) recombinants/5 \(\times 10^5\) recipient cells/217 bases of homology. These frequencies can be compared with internal controls for donor of a second unrelated, intact marker to measure the fraction of all DNA transformants that have undergone a recombination event between the tk markers. Data for Hind III linearized molecules show that one of every 10 cells transformed has included a functional tk recombinant. Southern blotting analysis of the tk\(^+\) transformants confirmed the presence of an intact tk gene, which must be the result of a homologous recombination event in the recipient cell during DNA transfer.

Additional experiments are directed toward developing conditions under which homologous recombination between donor sequences and their chromosomal homologues is sufficiently frequent that it can be used for site-directed introduction of cloned or synthetic sequences into the proper chromosomal locations. Cell lines, each of which contains one of the tk fragments, have been made. Each of these cell lines will be transformed and assayed by one of the two assays described above. As noted earlier, there are significant differences in the way various molecules, such as DEAE-dextran, calcium phosphate, or poly-L-ornithine stimulate DNA uptake and chromosomal integration. In part this may reflect different mechanisms of recombination induced by these molecules, and each of these molecules is being examined with respect to homologous recombination between donor DNA and the host cell chromosome.

100. CLONING OF HUMAN HMG CoA REDUCTASE AND OTHER GENES INDUCED BY CHOLESTEROL STARVATION

Investigators: Elliot C. Altman, James Mayhugh*

The immediate objective of this project was to isolate cDNA clones representing gene products induced by growing human fibroblasts under a cholesterol-depleted regimen. A limited number of gene products are known to be substantially induced under these conditions including HMG CoA reductase, the low density lipoprotein receptor and HMG CoA synthase. Cloned DNA probes for these genes are required in order to meet the long-term goals of understanding their complex pattern of regulation that ultimately results in cholesterol homeostasis. To this end, a cDNA library was constructed from the mRNA of cells highly induced for reductase and LDL receptor activity by growth in lipid-depleted medium containing high doses of mevinolin (a competitive inhibitor of HMG CoA reductase). These cells had, in fact, been selected over a long period of time to withstand growth in levels of mevinolin that are toxic to wild-type cells. It was hoped that these cells would provide an enriched source of the relevant RNAs, and might also shed light on the consequences of gene dosage alteration if the basis of mevinolin resistance was gene amplification. In retrospect, it appears that amplification of reductase does indeed play a role in mevinolin resistance in these cells, but is not sufficient in magnitude to account entirely for the level of drug resistance (or enzyme activity) observed. This will be studied in greater detail during the coming year.

Differential screening of this cDNA library has yielded several sequence families which are induced upon growth in mevinolin and suppressed upon growth in the presence of rich exogenous cholesterol sources. Experiments are in progress to identify the gene products represented by these cDNA clones.

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101. INDUCIBLE IMMUNOGLOBULIN HEAVY CHAIN SWITCHING

Investigator: Stuart K. Kim

Immunoglobulin C\(_H\) switching occurs when large regions, up to 200 kb, of the heavy chain locus are deleted. One end of the deletion is located between the \(V_H\) gene and the first \(C_H\) gene. The other end of the deletion is located slightly upstream of a downstream \(C_H\) gene. The effect of the deletion is to replace the first \(C_H\) gene with another \(C_H\) gene and thus switch the constant region of the expressed immunoglobulin. The ends of the deletion occur in regions denoted switch regions. Mark
Davis (Stanford University) and I previously investigated the mechanism of $C_H$ switching by isolating distinct switch rearrangements from different plasmacytomas. The recombinational breakpoints for $C_H$ switching may vary by over a kilobase. However, in each case, the sequences surrounding the recombinational breakpoints are similar. For example, the alpha switch region has 17 repeats of a particular 30·bp sequence and almost all recombinational breakpoints occur within these repeats. One explanation of these data is that these repeats serve as recognition sites for the $C_H$ switch recombinational enzymes.

I would like to introduce appropriately constructed immunoglobulin class switching vectors into B cells that are rearranging their endogenous immunoglobulin genes. For example, one vector has sequences in the order 5'-pBR322-SV40 ori-neo-Sµ-SV2GPT-Sγ2b-3'. Transfection of this vector into 1881, a B cell line which spontaneously switches isotype from IgM to IgGγ2b, will yield neo<sup>+</sup>, gpt<sup>+</sup> transformants. Immunoglobulin class switching should recombine the Sµ and Sγ2b region, delete the SV2GPT gene and express a neo<sup>+</sup>, gpt<sup>-</sup> phenotype. These cells can be isolated by their resistance to 6-thioguanine and the vector can be reisolated in bacteria by using the SV40 ori and pBR322 sequences. Schemes such as the one outlined above should help further elucidate the mechanism of $C_H$ switching by allowing much more rapid analysis of $C_H$ switch recombinants as well as in vitro mutagenesis of $C_H$ switch regions.

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102. ONCOGENE ACTIVATION IN MURINE PLASMACYTOMAS

Investigators: Stuart K. Kim, Leroy E. Hood

While working on normal immunoglobulin genes, aberrantly rearranged immunoglobulin genes were repeatedly isolated. These clones had a novel sequence located to the 5' side of the $C_a$ gene. Using mouse-hamster somatic cell hybrids, it was observed that the novel sequence derived from chromosome 15 whereas $C_a$ originated from chromosome 12. Thus, these clones span a translocation breakpoint. Next, it was observed that the novel sequence contained the cellular homologue of the v-myc oncogene. Since v-myc, derived from the retrovirus MC29, can cause avian tumors, and since almost all murine plasmacytomas contain a rearranged c-myc, it appears that the c-myc translocation causes the c-myc gene to become oncogenic and contribute to neoplasia.

A major roadblock in the study of the c-myc gene has been a lack of a biological assay. For example, using the cloned genes one can list all of the differences between the normal and rearranged c-myc genes in terms of how much is transcribed and changes in the primary protein structure. However, one still does not know the function of either the normal or rearranged c-myc or which of the changes in the gene actually unmasked its oncogenic potential. Thus, instead of further characterizing c-myc gene structure, and because the c-myc gene is unable to confer a transformed phenotype when introduced into mouse NIH3T3 cells, we would like to introduce the cloned, rearranged c-myc gene into normal lymphocytes. Evidently, plasmacytomas are the final result of several accumulated oncogenic mutations. For example, the plasmacytoma NSI is presently known to have a translocated c-myc gene, a rearranged c-mos gene, an activated B lym gene and a $C_K$ translocation of unknown function. It would be interesting to separate the steps of oncogenesis by engineering a lymphocyte that contained only the rearranged c-myc gene. The goal of this line of research is to outline the normal c-myc function and to delineate the cause of its oncogenic activation.

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103. LDL RECEPTOR GENE CLONING

Investigators: Paul K. Herman, Charles Reel

cDNA libraries have been constructed from the RNA of human and bovine cells (both cell types express relatively high levels of LDL receptor). Our immediate goal is to obtain a cDNA probe for the receptor, with regulatory and structural studies of the receptor being the long-range objective. The phage λ cloning vector λgt11 (developed and kindly provided by R. Young and R. Davis, Stanford University) has been used. It provides for the expression of fusion proteins consisting of parts of E. coli β-galactosidase along with protein encoded by the cDNA inserts.
Such fusion proteins are often recognized by antibodies raised against a purified cellular protein. In our case both monoclonal and polyclonal antisera against the LDL receptor will be used to screen the expression libraries. We are also investigating the possibility of "ligand screening" based on the observation that LDL receptors (M. S. Brown, personal communication) (and several other receptors as well) will still bind their respective ligands after the receptor has been SDS treated and affixed to a solid matrix.

*Undergraduate, California Institute of Technology.

104. CHIMERIC GENES CONSTRUCTED TO ASSAY REGULATION

Investigator: Paul R. Mueller

The 5' flanking region of genes has been shown to be sufficient for appropriate gene regulation events (Mayo et al., 1982; Robins et al., 1982). In these studies, the 5' flanking or promoter region of a regulated gene is fused to the transcription unit of an "indicator" gene which is not ordinarily regulated. Upon introduction into an appropriate host cell, the indicator gene product is produced under the regulatory control of the gene donating its 5' promoter region and 3' non-coding sequence.

I have made two similar constructions in which the promoter regions of β-interferon or metallothionein are fused to the protein coding region of dihydrofolate reductase (DHFR). Under normal conditions, β-interferon can be induced by a variety of agents including poly(rI) and poly(rC), and metallothionein can be induced by heavy metals such as cadmium. When my constructions are transformed into appropriate cell types, DHFR should be produced upon induction by these agents. DHFR is the indicator of choice because it can be quantitated easily by several methods: (1) measuring the ability of a cell extract to reduce folic acid; (2) binding 3H-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.

Cell lines which are stably transformed with each of these constructions will be screened for mutants which are constitutive up or down producers of DHFR, which could be caused by mutations either cis or trans to the indicator construct. A cis mutation would help identify sequences important to promoter function, whereas a trans mutation would help identify new genes involved in regulating the response. These chimeric gene constructions will also serve to conveniently assay the effects of cis-acting control elements referred to as "enhancers."

References:
Summary: Flagellated bacteria possess a remarkable motile 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 cell 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. The nature of the signal that couples the receptors to the motors is not known. 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.

Some bacteria have no recognizable organelles of locomotion, yet move steadily when in contact with solid surfaces. We would like to understand the mechanism for this gliding motility.

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.

ADAPTATION IN BACTERIAL CHEMOTAXIS

Investigator: Steven M. Block

Bacteria, in their sensory behavior, exhibit a feature that is common to most living organisms—they adapt. Cells are able to ignore the ambient level of sensory input and respond to changes about that level.

Cells of Escherichia coli, tethered to a glass by a single flagellar filament, were subjected to constant flow of a medium containing the attractant α-methyl-D,L-aspartate (Block et al., 1983). Its concentration was varied with a programmable mixing apparatus over a range spanning the dissociation constant of the chemoreceptor at rates comparable to those experienced by cells swimming in spatial gradients. When an exponentially increasing ramp was turned on (a ramp that increases the chemoreceptor occupancy linearly), the rotational bias of the cells (the fraction of time spent spinning counterclockwise) changed rapidly to a higher stable level, which persisted for the duration of the ramp. The change in bias increased with ramp rate, i.e., with the time rate of change of chemoreceptor occupancy. This behavior can be accounted for by a model for adaptation involving proportional control, in which the flagellar motors respond to an error signal proportional to the difference between the current occupancy and the occupancy averaged over the recent past. Distributions of clockwise and counterclockwise rotation intervals were found to be exponential. This result cannot be explained by a "response regulator" model, in which transitions between rotational states are generated by threshold
crossings of a regulator subject to statistical fluctuation (Koshland, 1977); this mechanism generates distributions with far too many long events. However, the data can be fit by a model in which transitions between rotational states are governed by first-order rate constants. The error signal acts as a "bias regulator," controlling the values of these constants.

References:

106. THE CHEMOTACTIC IMPULSE RESPONSE

Investigators: Jeffrey E. Segall, Steven M. Block

The chemotactic behavior of E. coli was studied by exposing tethered cells to pulses of chemicals delivered iontophoretically (Block et al., 1982). Normally, wild-type cells spin alternately clockwise (CW) and counterclockwise (CCW), changing direction about once per second. When cells were exposed to a very brief diffusive wave of attractant, the probability of spinning CCW quickly peaked and then fell below the pre-stimulus value, returning to baseline within a few seconds; repellent responses were similar but inverted (see the figure on p. 77 of Biology 1982). The width of the response indicates that cells integrate sensory inputs over a period of seconds, while the biphasic character implies that they also take time derivatives of these inputs. The sensory system is maximally tuned to concentration changes that occur over a span of about 2 sec, an interval over which changes normally occur when cells swim in spatial gradients; it is optimized to extract information from signals subject to statistical fluctuation.

Impulse responses of cells defective in methylation were similar to those of wild-type cells, but did not fall as far below the baseline, indicating a partial defect in adaptation. Impulse responses of cheZ mutants were aberrant, indicating a serious defect in excitation. Impulse responses of strains containing other mutations that affect signal processing are currently being analyzed. In this way, we hope to determine the role played by proteins known to be involved in bacterial chemotaxis.

Reference:

107. COORDINATION OF FLAGELLA ON FILAMENTOUS BACTERIAL CELLS

Investigators: Akira Ishihara, Jeffrey E. Segall, Steven M. Block

Video techniques were used to study the coordination of different flagella on single filamentous cells of E. coli (Ishihara et al., 1983). Filamentous, nonseptate cells were produced by introducing a cell-division mutation into a strain that was polyhook but otherwise wild type for chemotaxis. Markers for its flagellar motors (ordinary polyhook cells that had been fixed with glutaraldehyde) were attached with antihook antibodies. The markers were driven alternately clockwise and counterclockwise, at angular velocities comparable to those observed when wild-type cells are tethered to glass. The directions of rotation of different markers on the same cell were not correlated; reversals of the flagellar motors occurred asynchronously. The bias of the motors (the fraction of time spent spinning counterclockwise) changed with time. Variations in bias were correlated, provided that the motors were within a few micrometers of one another. Thus, while the direction of rotation of flagellar motors is not controlled by a common intracellular signal, their biases are. This signal appears to have a limited range, shorter than would be expected for an electrical potential.

To learn more about the nature of this signal, we are delivering chemicals iontophoretically at different points along the length of filamentous cells and observing the size of the responses indicated at one or more markers.

Reference:

108. INDUCTION OF ROTATION IN PARALYZED BACTERIAL MUTANTS

Investigators: Steven M. Block, Christopher Kaiser*

When wild-type cells of E. coli are mutagenized
and selected for loss of motility, two types of mutants are found. Cells of the first type have lost the ability to synthesize one or more parts of the flagellar apparatus; they lack the necessary equipment to swim. Cells of the second type produce intact flagella but are unable to rotate them; they have a paralyzed phenotype. Cells bearing this latter phenotype map in one of two alleles of the mot gene, designated as A and B. Expression of mot is controlled, in part, from an operon coding for motA, motB and cheA, termed the "mocha" operon (Silverman and Simon, 1976). Flagellar basal bodies isolated from mot− cells appear identical to those of the wild type, both in morphology and protein composition. The mot genes appear to code for the synthesis of polypeptides (MW=31,000 and 39,000 for A and B, respectively) that are not directly linked to the basal body, but are found instead in the cell membrane fraction. Their synthesis is required for flagellar rotation. It has been found that when paralyzed cells are infected with a λ-E. coli hybrid phage carrying motA, swimming ability is restored within minutes, "resurrecting" bacteria that lay dead in the water (Silverman et al., 1976). We are performing analogous experiments using a tethered-cell system. In our case, mot synthesis is directed in a motB− strain by a plasmid carrying the motB gene fused to the promoter region of the lac operon and consequently under lac control (plasmid pGM1; Boyd et al., 1982). Additional modifications of this strain have been performed to ensure that mot synthesis is turned off almost completely prior to induction. After the addition of an inducer, such as IPTG, tethered cells are observed to begin rotation after times as short as five minutes. They start abruptly with a slow, almost constant angular velocity, generally in the CCW mode. Reversals can occur quite soon after the start of rotation. After a short period of rotation at this slow speed, and in the continued presence of an inducer, cells abruptly increase their rotation rate by roughly a factor of two, stably maintaining the increased speed for a time. Subsequent steps in rotation rate also have been observed; cells continue to increase in speed until their rate is comparable with energized wild-type cells (typically 5-10 Hz). Since the proteins specified by the mot genes may represent ion channels or force-generating units associated with the rotary motor, we are currently performing experiments designed to follow motB synthesis and incorporation in these cells. Results from these experiments may provide information about the function of motB and allow tests of current theories of motor function.

References:

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109. PHOTODYNAMIC MODIFICATION OF THE FLAGELLAR MOTOR

Investigator: M. Patricia Conley

As noted in last year's annual report (Biology 1982, No. 105), the photodynamic dye eosin inhibits motility in Streptococcus strain V4051, probably by acting on the flagellar motor directly, reducing its complement of independent force generators. To rule out the alternative possibility that photoxidation impairs motility indirectly by reducing the energy supply (the protonmotive force), we measured the membrane potential (Δψ) attained in cell suspensions energized artificially with a potassium diffusion potential in the presence or absence of eosin. The membrane potential was determined from the distribution of tracer amounts of Rb86. While cells exposed to eosin and light developed a slightly smaller Δψ than that of control cells, the difference was not great enough to account for the corresponding marked reduction in speed.

As a further test of the hypothesis that the motor has independent force generators and that their number is reduced by photodynamic oxidation, we measured the rates of rotation of tethered cells as a function of protonmotive force and studied the changes generated in this dependence by exposure to eosin and light. Treated cells had a larger threshold for rotation and a smaller change in rate per
unit change in protonmotive force than control cells, as predicted by the hypothesis. Thus, it appears that flagellar motors can run at reduced power, like an internal combustion engine running on only a few of its cylinders. These results are consistent with those described in Abstract No. 111.

110. SYMMETRY OF THE BACTERIAL FLAGELLAR MOTOR

Investigator: Markus Meister

Electron micrographs of detached rings from the motor of E. coli suggest a rotational symmetry of 16 (DePamphilis and Adler, 1971). In freeze-etched preparations of Aquaspirillum serpens, one can see circlets of 14 to 16 studs surrounding depressions that presumably contained such rings (Coulton and Murray, 1978). Is this rotational symmetry reflected in the operation of the flagellar motor?

Frequency analysis of the rotational motion of tethered cells of E. coli has shown no such periodicity; however, these dynamical measurements were impaired by low-pass filtering of the torque generated by the motor due to torsional elasticity of the flagellum and frictional drag on the cell body (Berg et al., 1982). I would like to carry out static measurements and determine whether the bacterial motor stops at discrete positions.

Tethered cells of Streptococcus can be started and stopped repeatedly, either by energizing starved cells via a potassium diffusion potential or by de-energizing metabolizing cells with the uncoupler dinitrophenol. A photomicrograph of the field is taken each time the cells stop. The angular positions at which each cell stops in each of the subsequent photographs can be measured to within ±2° using a modified dissecting microscope. These angles are analyzed using a Fourier transform technique in order to find periodicities. Preliminary results suggest 5 or 6 discrete stopping positions in the motor of Streptococcus. The experiment is now being done on E. coli.

References:

111. CHEMIOSMOTIC COUPLING TO THE BACTERIAL FLAGELLAR MOTOR

Investigators: Shahid M. M. Khan, Howard C. Berg

The torque generated by the flagellar motor of Streptococcus strain V4051 was determined from rates of rotation of tethered cells in media of different isotopic composition and temperature (Khan and Berg, 1983). Starved cells were energized artificially with either a potassium diffusion potential or a pH gradient. The torque increased linearly with protonmotive force. Identical results were obtained in media made with D2O or H2O; there was no solvent isotope effect. At a fixed protonmotive force, the torque was approximately constant over a temperature range of 4 to 36°C. In cells chemotactically inert to changes in cytoplasmic pH, the motor turned counterclockwise when protons moved inward and clockwise when they moved outward. These results suggest that the motor is a reversible engine driven by simple acid-base dissociation. The model for the motor discussed in last year's annual report (Biology 1982, No. 103) is consistent with these and a variety of other results (Berg and Khan, 1983).

References:

112. FURTHER TESTS OF A MODEL FOR THE FLAGELLAR ROTARY MOTOR

Investigators: Shahid M. M. Khan, Markus Meister

The model referred to in Abstract No. 111 has led to specific predictions about the mean torque and variations in torque generated at different proton gradients, at different absolute values of pH, and at different external viscosities. Tests of these predictions are in progress.

113. CHEMIOSMOTIC COUPLING TO THE BACTERIAL MEMBRANE ATPase

Investigators: Shahid M. M. Khan, Howard C. Berg

In an effort to learn whether other chemiosmotically-powered devices might be driven by
acid-base dissociation, we studied rates of ATP synthesis by the proton-translocating ATPase of the motile Streptococcus strain V4051 (Khan and Berg, 1983). Starved cells were energized artificially by exposing their membranes to a variable electrical potential difference (internal medium negative) and a fixed pH difference (internal medium alkaline). The initial rates of ATP synthesis increased exponentially with protonmotive force. The results were the same in D$_2$O and H$_2$O; there was no solvent isotope effect. At a fixed protonmotive force, the rates were strongly dependent on temperature, as expected for a reaction with a large enthalpy of activation. At a different protonmotive force, the rates varied with temperature in an identical fashion; there was no change in the enthalpy of activation. We conclude that protonation-deprotonation steps are not rate limiting and that the protons that cross the membrane drive ATP synthesis by mass action. The transmembrane electric field acts by changing the concentrations of the reactants, not by changing the configuration of the enzyme-substrate complex.

Reference:

114. THE AVOIDANCE RESPONSE IN PHYCOMYCES

Investigator: Paul W. Meyer

A Phycomyces sporangiophore (spph) bends away from any object placed within a few millimeters of its growing zone. The following two results will help us determine the physical mechanism that allows the spph to detect such an object.

First, we have found that the avoidance rate for different spphs varies by less than ±10% if a 22 mm diameter glass disk, cleaned in nitric acid, is placed 1 mm away from the growing zone. Such precision is obtained when the ambient temperature and relative humidity are controlled to within ±1%. The ambient air speed is kept below 5 µm/sec, the age of the mycelium is between 120 and 150 hours, the initial orientation of the spph is toward the disk at an angle of 2° or more, and the avoidance rate is corrected for growth rate and aiming error. Under these conditions, the avoidance rate falls off sharply as the relative humidity is increased above 90%; at 99% r.h. the spph avoids the glass disk only half as fast as at 76% r.h.

Second, we have found that if the experiment is done in an airtight aluminum and glass chamber (volume 20 cm$^3$), with all of the inner surfaces first cleaned in alkaline detergent, the spph no longer avoids the disk. The avoidance response returns when an adsorbent material, such as an acid-cleaned glass fiber filter or a piece of Kimwips, is placed in the chamber. We are in the process of trying to find out what it is that is adsorbed.

115. GLIDING MOTILITY OF CYTOPHAGA

Investigators: I. Richard Lapidus, Howard C. Berg

Cells of Cytophaga strain U67, a gram-negative rod-shaped bacterium, exhibit a number of motile behaviors. Individual cells glide on glass at speeds in excess of 1 µm/sec. They stop, back up, pivot, gyrate and rotate about one pole. They propel polystyrene latex spheres and other objects along their surfaces. The velocity of gliding cells and of the spheres propelled along their surfaces is independent of external viscous drag over a very wide range (a factor of 100). The motility is maintained over a large temperature span (5°-40°C). These behavioral phenomena have been analyzed quantitatively using video techniques. The data are consistent with a model in which adsorption sites on the cells move within the fluid outer membrane along tracks fixed to the rigid peptidoglycan framework (Lapidus and Berg, 1982). Electron microscopy is being used in an effort to visualize the binding sites and the tracks.

References:

Publications
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 in flagella and muscle.

Professor: Charles J. Brokaw
Research Fellows: Charlotte K. Omoto, Lee K. Opresko
Research Staff: Sandra M. Nakada, Scott N. Richman*
*Undergraduate, California Institute of Technology

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116. BENDING PATTERNS OF CHLAMYDOMONAS FLAGELLA

Investigators: Charles J. Brokaw, David J. Luck*

The use of a mutant, uni-1, has enabled us to obtain cells of Chlamydomonas that develop only one flagellum. The normal asymmetric bending pattern of this flagellum causes the cells to spin around a point on the microscope slide and allows us to obtain good photographic records of the flagellar bending pattern. These techniques enable us to characterize precisely the bending patterns of flagellar motility mutants. However, our work during the past year has concentrated on further characterization of the wild-type bending patterns.

The normal asymmetric bending pattern used for forward swimming was studied in flagella ranging in length from 5 to 15.5 µm, by observing cell samples at different stages in flagellar growth. There are only small changes in wavelength and curvature associated with changes in length; these changes are not sufficient to maintain similarity of the bending pattern at a different scale. In addition to the asymmetric forward mode bending pattern, Chlamydomonas has a reverse mode that is elicited by light in the presence of Ca²⁺ ions. In the reverse mode, the flagellum propagates symmetrical, planar, undulatory waves with a shear amplitude that is the same as in the forward mode; there is a 19% increase in beat frequency and a similar decrease in wavelength. There is a reorientation of flagellar beat direction towards the axis of the cell in the reverse mode that is caused both by the decrease in asymmetry of beat and by activation of
sliding in principal bends at an earlier time in the beat cycle, relative to the time of activation of sliding in reverse bends. There are additional rare modes of beating having larger shear amplitudes that may be related to intermediate stages in the transition between forward and reverse beating modes.

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117. REACTIVATION OF CHLAMYDOMONAS FLAGELLA
Investigators: Charlotte K. Omoto, Charles J. Brokaw

Techniques for demembranation and ATP-reactivation of Chlamydomonas have been modified to allow us to photograph reactivated uni-1 cells in a manner similar to intact cells. Demembranation and reactivation allow us to control and change various ionic conditions and nucleotide concentrations. We have studied two factors which markedly affect the motion of many flagella, including those of Chlamydomonas: ATP and Ca²⁺. At low Ca²⁺ concentration (<10⁻⁶ M), the waveform is indistinguishable from the forward mode of intact flagella. This waveform is almost independent of ATP concentration over the range from 1 mM to 0.10 mM. Beat frequency with change in ATP concentration, for Chlamydomonas, like other axonemes, displays simple saturation kinetics with maximum beat frequency of around 50 Hz and Kₘ of 0.15 mM. At high Ca²⁺ (>10⁻⁴ M), many flagella are released from the cell but display symmetrical waveform and an increase in beat frequency similar to the reverse best of intact flagella. At intermediate calcium concentrations, there is marked decrease in the percentage of motile flagella with very little motility at 10⁻⁵ M. Photography of flagella on demembranated uni-1 cells has allowed us to find two new beat patterns at intermediate calcium concentrations. One is a symmetrical, undulatory bending pattern that resembles the reverse bending pattern, except that the asymmetry in time of initiation of principal and reverse bends is absent. Consequently, the flagellum does not reorient toward the reverse position properly, and the cell rotates slowly rather than swimming straight. The other pattern is a low-amplitude asymmetric bending pattern, which also causes slow rotation. We have not seen any large-amplitude patterns resembling the rare modes of beating seen with undemembranated flagella.

118. ACTIVATION OF NON-MOTILE FLAGELLA
Investigator: Charles J. Brokaw

Spermatozoa from the tunicate, Ciona, diluted directly into a Triton-demembranation solution and subsequently exposed to MgATP, assume a quiescent configuration and do not show reactivated motility. An activation process is required in order to obtain reactivated motility. In vivo activation can be obtained by diluting live spermatozoa into seawater solutions containing theophylline; subsequent demembranation produces ATP-reactivable spermatozoa. In vitro activation can be obtained by incubating demembranated spermatozoa with cAMP or with the catalytic subunit of a cAMP-dependent protein kinase. cAMP activation is very sensitive to dilution of the demembranated sperm sample, as expected if it is dependent upon a cAMP-dependent kinase in the Triton supernatant. Protein kinase activation is more resistant to dilution, but can be inhibited by thorough washing of the demembranated spermatozoa and restored by addition of Triton supernatant. This observation suggests that the supernatant contains a regulatory component in addition to a protein kinase, but its identity is not yet established.

Some preparations of spermatozoa from the sea urchin, Lytechinus, show only 10-20% reactivation of motility after demembranation. 100% reactivation of motility can be obtained if these spermatozoa are incubated with cAMP under conditions similar to those used for in vitro activation of Ciona spermatozoa. It has not been possible to obtain sperm preparations from the sea urchin Strongylocentrotus purpuratus that show a cAMP requirement for reactivated motility.

119. cAMP-DEPENDENT PHOSPHORYLATION ASSOCIATED WITH ACTIVATION OF MOTILITY OF CIONA SPERM FLAGELLA
Investigators: Lee K. Opresko, Charles J. Brokaw

During in vitro activation experiments, the
presence of cAMP causes a greater than fivefold enhancement of $^{32}$P incorporation by demembranated Ciona spermatozoa. Analysis by one-dimensional PAGE and autoradiography shows many axonemal protein bands that become labeled during in vitro activation with cAMP or protein kinase. The labeling of some of these bands is specifically reduced by in vivo activation of motility before demembranation, suggesting that these proteins also become phosphorylated during in vivo activation of motility. These phosphorylated proteins appear to include dynein heavy chain components, but axonemal tubulin is not phosphorylated. Partially phosphorylated spermatozoa can be activated by an increase in KCl concentration that appears to dissociate one phosphorylated component from the axoneme.

120. AXONEMAL BENDS AND TWISTS IN THE QUIESCENT STATE OF CIONA SPERM FLAGELLA
Investigators: Charlotte K. Onoto, Charles J. Brokaw

A simple planar model of sliding microtubules can predict the location and amount of sliding required to produce a certain planar bend. The accuracy of this prediction relies on the assumption that no twists occur in the axoneme. However, previous studies indicated that twists may occur.

Using platinum replicas of demembranated axonemes, twists were directly visualized and quantitated on planar axonemes. Preliminary results using this method also showed that there were twists. However, analyses of platinum replicas of the S-shaped quiescent state of Ciona spermatozoa showed opposite direction of twist depending on the orientation of this S-shape on the substrate. Detailed analyses suggest that most, if not all, of the twists observed are due to the artifact of a curved shape settling onto a surface. If twists do occur in quiescent sperm, they are calculated to be less than 0.4 radian. This small amount of twist will not significantly affect the amount of sliding predicted to produce the waveform assuming no twists.

Previous reports of twists are similar to those found on quiescent Ciona axonemes and are consistent with the interpretation that most if not all the twists observed are due to a curved shape flattening onto a substrate.

121. BENDING WAVE PROPAGATION BY A DISTRIBUTED RELAXATION OSCILLATOR

Investigator: Charles J. Brokaw

Flagellar bending is generated by an active shear mechanism that operates between the outer microtubular doublets. It has been suggested that the active shear mechanism is controlled by the curvature of the flagellum, establishing a feedback loop that causes oscillation and bend propagation. Computer simulations have demonstrated that such oscillation and bend propagation will occur, but a model with completely realistic behavior has not been achieved.

One of the simplest specifications of control by curvature, which has been used in some of our flagellar modeling, states that the direction of operation of the active shear mechanism is switched when the local curvature of the flagellum attains a critical magnitude, $K_0$. This specification essentially creates a relaxation oscillator, with the curvature at any point on the flagellum oscillating between $+K_0$ and $-K_0$, but the properties of the oscillator depend on behavior of the active shear system throughout the length of the flagellum, not just at the point of interest. This oscillatory mechanism can be studied in a simplified flagellar model containing only two elements:

1) An elastic bending resistance that produces a resistive bending moment equal to a constant times the curvature.

2) An active shear mechanism that generates a longitudinal shear force between microtubular doublets, and has the following properties. When subjected to a rapid shear displacement, the force changes proportionately, as if the force is generated by a linear elastic shear resistance. Following such a displacement, the force recovers to its original value with an exponential time-course. Because of these properties, the force during steady-state sliding will decrease linearly with sliding velocity; the system is therefore a rough analog of cross-bridge systems suggested for the generation of sliding in muscle and flagellae. This simplified system generates propagated
bending waves similar to those obtained with other flagellar models, even though no external viscosity is considered. Analytical evaluation of this system yields a solution curve in the frequency-wavelength plane, but does not identify a specific point on the curve. Until we can understand why a particular solution is obtained from the numerical simulation of the system, we cannot decide whether the unrealistic performance of the computer models of flagella is the result of an unrealistic mechanism for controlling the active sliding, or an unrealistic mechanism for determining a particular solution.

**Publications**


**Summary:** The general area of interest is the chemical 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. Areas in which studies have been made include: the relationship between protein structure and local group chemical kinetics of home proteins; the mechanism of biological electron transfer processes; the problem of accuracy in the synthesis of biological molecules—its limits, mechanisms to enhance accuracy, and its origin; and emergent properties of interacting neurons.

**Emergent Properties of Neural Networks**

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 model systems of 30-1000 neurons to examine what biologically useful collective properties spontaneously arise. Several such properties do occur of which the most obvious is the interaction...
between different aspects of a single memory. In the model system, this results in the ability of the network to reconstruct all of a particular memory from any sub-part sufficiently large to distinguish that memory from other stored memories. Learning algorithms, the effects of unlearning, the dynamics of network decision-making, the relation of the model to more realistic properties of neurons, and physical realizations of a model of collective activities are being pursued.

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123. THE DYNAMICS OF CO BINDING TO HEME PROTEINS
Investigators: Noam Agmon, John J. Hopfield
The kinetics of ligand binding to heme proteins depends on the structure of the protein. While at high temperatures, the protein structure fluctuates rapidly and the rate of binding is averaged over these fluctuations; at low temperature the fluctuations are very slow, and binding takes place for a distribution of different structures. We have constructed a detailed energy surface model on which to calculate the dynamics of ligand binding, and have used it to describe some of the experiments of Hans Frauenfelder and coworkers at the University of Illinois, Urbana. The theory unifies a considerable body of experiments in a simple structure-based model, and has predicted results for a new class of experiments which have yet to be tried on the basis of the old results.

124. ELECTRON TRANSFER PROCESSES
Investigators: David N. Beratan*, George Celler, Alvin D. Joran*, Peter Dervan*, John J. Hopfield, Jose Nelson Onuchic, Burton A. Leland
A major limitation to the understanding of the electron transfer processes central to bioenergetics has been the absence of detailed structure for any biological system of known electron transfer properties. Feeling that we perhaps understand the general constructs which must be essential to the occurrence and control of electron transfers in bacterial photosynthesis, we have embarked on the synthesis and physical study of a series of electron transfer molecules. The design of these molecules incorporates the ideas believed essential to the charge separation process. The first target molecule is

The electron transfer rate for this particular molecule is also being calculated theoretically. At the same time, there are persistent experimental suggestions that the conventional theoretical formulation is wrong in certain limiting cases, and an investigation of the fundamental hypothesis behind the conventional theory is under way.

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PUBLICATIONS

Associate Professor: Elias Lazarides
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Summary: The development of cell shape is a problem of cellular morphogenesis and pattern formation during differentiation and is ultimately involved with and tailored to a cell's physiology. The fundamental role that cytoplasmic filaments, and in particular actin filaments and intermediate filaments, play in this process has become apparent over the past several years from our studies on the composition, expression and assembly of these two filament systems during differentiation. Two cell types that have contributed to our realization of the involvement of these two-filament systems in cellular morphogenesis are the nucleated chicken erythrocyte and skeletal muscle. The development of new techniques for studying the structural topology of these cells has revealed unexpected similarities in their structure and has offered a unique opportunity to investigate at the molecular level the regulation of the expression and assembly of the different filament proteins during differentiation and the similarities and differences between these two cell systems. Similar to mammalian erythrocytes, the nucleated chicken erythrocyte contains a two-dimensional submembranous network of filaments responsible for conferring structural support and deformability to the cell as well as restricting the lateral mobility of certain transmembrane polypeptides. A principal component of this network is the high molecular weight protein spectrin. Spectrin is a heterodimer composed of equal amounts of two subunits α and β. Spectrin interacts with actin and with the plasma membrane. Association of the spectrin-actin network with the plasma membrane is mediated by the protein ankyrin (goblin in chickens) which interacts simultaneously with the β subunit of spectrin and with a subset of the transmembrane anion transporters. Superimposed on this spectrin-actin network, and in association with it, is a system of intermediate filaments which forms a three-dimensional network and extends from the nucleus to the spectrin network on opposite sides of the cell. In this fashion the spectrin network and the intermediate filament network form a three-dimensional transcytoplasmic matrix which may mechanically integrate the plasma membrane and the nucleus. Principal components of the intermediate filament network are the protein vimentin, as the core filament protein, and the high molecular weight protein, synemin, as a peripherally associated component. Immunelectron microscopy has shown that in adult erythrocytes one of the functions of synemin is to crosslink vimentin filaments at periodic intervals, thus presumably increasing the tensile strength of this transcytoplasmic matrix. In addition, the equator of the football-shaped chicken erythrocytes contains a marginal band composed of a bundle of microtubules which is also superimposed and in association with the spectrin-actin network, but spatially segregated from intermediate filaments. Studies with adult skeletal muscle have unexpectedly revealed that this cell type also contains a transcytoplasmic matrix composed of spectrin-actin and intermediate filaments. In adult muscle this matrix is present perpendicular and at right angles to the myofibrils and specifically at the peripheries of the Z-disc component of myofibrils. This matrix appears to link myofibrils laterally to each other and ultimately to the plasma membrane. On the cytoplasmic side of the plasma membrane this matrix is coextensive with a system of intermediate filaments that runs along the length of the muscle fiber in close association with the plasma membrane and which is interconnected by submembranous rings.
of spectrin at the level of the Z-line and the M-line of the myofibrils. Surprisingly, the composition of both spectrin and intermediate filaments in adult muscle is very similar to that in erythrocytes, further suggesting a similarity in the structure of this transcytoplasmic scaffold in the two cell types. In mature muscle, spectrin is composed of two subunits, \( \alpha \) and \( \beta \), antigenically related to the two spectrin subunits in erythrocytes. Intermediate filaments contain both vimentin and synemin, but in addition they contain a muscle-specific filament subunit, desmin. The presence of synemin at the peripheries of Z discs suggests that, by analogy to its function in erythrocytes, synemin may also crosslink vimentin and desmin-containing filaments at the peripheries of Z discs. As spectrin is an actin filament crosslinking protein, this spectrin-actin intermediate filament-based scaffold may function as a highly noncovalently crosslinked matrix responsible for integrating myofibrils with each other and with the plasma membrane both along the long axis of the fiber and transversely at the level of the Z- and M-lines.

Having established the composition and structural similarities of this matrix in muscle and erythrocytes, we are in a unique position to study the regulation of the expression and assembly of its different components during differentiation. Assembly of this matrix during erythroid cell development can be viewed essentially as a unidirectional process since this cell is immotile, does not undergo any gross transitions in its shape during development and the sole presumed function of this cytoskeletal matrix is to provide structural support to the cell as a whole. On the other hand, muscle cells have motility as their primary function and assembly of this transcytoplasmic matrix has to be coordinated with the expression and assembly of myofibrils. Furthermore, expression and assembly of these two components cannot be viewed simply as a unidirectional process since during differentiation, the primitive cell, the myoblast, has to change and substitute its own cytoskeletal elements upon fusion with those of the differentiating muscle fiber. The abstracts that follow represent the studies we have carried out over the past year aimed at elucidating the regulation of the expression and assembly of spectrin and vimentin during erythroid and muscle differentiation.

125. A SINGLE CHICKEN VIMENTIN GENE CODES FOR TWO mRNA'S WITH TISSUE-SPECIFIC EXPRESSION

Investigators: Yassemi G. Capetanaki, John J. Ngai

In order to understand and determine in detail the biochemical and structural similarities of the various intermediate filament subunits and study their modes of expression as well as their evolutionary relationship, we have attempted to isolate and characterize their respective genes. So far we have isolated both genomic and cDNA clones for the intermediate filament subunit vimentin. The identity of these clones was confirmed by hybrid selection of the vimentin mRNA and subsequent two-dimensional electrophoresis of its in vitro translation products. Restriction analysis of the eight genomic clones obtained showed that all of them contain overlapping fragments of the same gene. Southern blot analysis has confirmed the observation of others (Dodemont et al., 1982; Zehner and Paterson, 1983) that vimentin is present in a single copy in the haploid chicken genome. However, RNA blot studies revealed that the single vimentin gene is transcribed into two different species of mRNA with molecular lengths of 2 kb and 2.3 kb. These RNAs possibly differ at least in the length of their 3' untranslated region (Zehner and Paterson, 1983). We have found cell-specific regulation of the expression of these mRNAs; whereas both mRNA species are present in muscle cells, fibroblasts, spinal cord, and lens, only the lower molecular weight mRNA is expressed in chicken embryonic erythrocytes obtained from 4, 10 and 15-day-old embryos. In addition, RNA blot analysis of different stages of cultured myogenic cells has revealed the expression of both mRNAs during myogenesis. We have subcloned the entire vimentin gene in pBR322 and we are studying in further detail its structure by S1-nuclease mapping, R-loop analysis, and sequencing.
References:

126. VIMENTIN FILAMENTS ARE ASSEMBLED FROM A SOLUBLE PRECURSOR IN AVIAN ERYTHROID CELLS

Investigator: Ingrid Blikstad

The spontaneous assembly of intermediate filament proteins into filaments has been extensively studied in vitro. However, the regulation of the assembly of intermediate filaments in vivo has received little attention. In this study, we have investigated the synthesis and assembly of the intermediate filament protein vimentin in chicken embryo erythroid cells. After various periods of 35S-methionine incorporation, cells were lysed in a Triton X-100-containing buffer and separated into a soluble and insoluble (cytoskeletal) fraction. Analysis of these two fractions by two-dimensional gel electrophoresis shows that vimentin is almost exclusively present in the cytoskeletal fraction and that newly synthesized vimentin is rapidly incorporated into this fraction. However, after a short pulse-labeling period, a prominent labeled protein at the position of vimentin is present in the soluble fraction. By immunautoradiography and immunoprecipitations with vimentin antibodies, this protein was identified as vimentin. The vimentin in the soluble fraction is not sedimented by high speed centrifugation, suggesting that it does not consist of short filaments. Following different pulse-labeling periods, assembly of newly synthesized vimentin in the cytoskeletal fraction increases linearly while the radioactivity in the soluble vimentin remains constant. During a 2-hour pulse-chase period, the vimentin in the soluble fraction is chased into the cytoskeletal fraction with a half life of 7 min. These results show that in chicken embryo erythroid cells, newly synthesized vimentin is rapidly assembled into filaments from a soluble precursor.

127. SYNTHESIS AND POSTTRANSLATIONAL ASSEMBLY OF SYNEMIN IN AVIAN ERYTHROID CELLS

Investigator: Randall T. Moon

Intermediate filaments in avian erythroid cells consist of a core filament of vimentin and at least one peripheral polypeptide, synemin, which binds vimentin filaments at regularly spaced intervals (Granger et al., 1982; Granger and Lazarides, 1982). Since assembled intermediate filaments are relatively insoluble under physiological conditions, two questions arise. First, are either newly synthesized vimentin or synemin cotranslationally assembled into the cytoskeleton, as has been suggested for cytoskeletal proteins in general (Fulton and Wen, 1983), or are they assembled posttranslationally? Second, what regulates synemin binding to vimentin filaments? The first question is partially answered by a recent study (Blikstad and Lazarides, 1982; see Abstract No. 126) which shows that vimentin is assembled posttranslationally from a soluble precursor. In the present study, the kinetics of synthesis and assembly of synemin relative to vimentin were investigated by labeling chicken embryo erythroid cells with 35S-methionine, followed by separating the cells into a detergent-soluble and detergent-insoluble (cytoskeletal) fraction. The amount of newly synthesized vimentin and synemin was then determined by immunoprecipitation of these labeled polypeptides, followed by gel electrophoresis and quantitative densitometry of fluorographs. The incorporation of labeled vimentin into the insoluble fraction increases linearly with time, while a small pool of soluble vimentin saturates quickly and hence displays a constant level of labeled vimentin. In contrast, synemin accumulates rapidly into a soluble pool, which, unlike vimentin's soluble pool does not saturate quickly, and synemin begins to accumulate in the insoluble fraction only after a considerable lag of time. Pulse-chase experiments reveal that the detergent-soluble pools of both vimentin and synemin contain precursors for the posttranslational assembly of intermediate filaments, and that the majority of the soluble polypeptides are assembled rather than degraded. Finally, immunoprecipitation of the detergent-soluble and insoluble fractions with synemin antiserum demonstrates that synemin and vimentin can be coprecipitated from the cytoskeletal fraction in the form of a complex, but not from the soluble fraction. This suggests that
synemin does not interact with vimentin in the soluble fraction. Since the data are inconsistent with previous ideas on the assembly of the cytoskeleton (Fulton and Wan, 1983), the following model was developed to explain the assembly of erythroid intermediate filaments. Newly synthesized vimentin first enters a small soluble pool, from which precursors are rapidly and post-translationally assembled into elongating detergent-insoluble intermediate filaments. Newly synthesized synemin also enters a soluble pool, but is not immediately assembled due to the limited availability of binding sites. We hypothesize that synemin binding sites are generated by higher order changes in vimentin filaments which arise during the course of vimentin filament elongation.

References:

128. EXPRESSION OF THE MAJOR NEUROFILAMENT SUBUNIT IN CHICKEN ERYTHROCYTES

Investigator: Bruce L. Granger

Intermediate filament polypeptides have emerged as potential markers for determining the embryonic origin of cells, following cells during development, and determining the cellular origin of tumors. Deviations from the general scheme of intermediate filament expression may reveal unforeseen relationships between different cell types as well as provide clues to the mysteries of intermediate filament function.

Neurofilaments are the intermediate filaments found in mature neurons; they are composed predominantly of a 70,000 dalton polypeptide (NF70) which forms the core polymer. NF70 is currently thought to exist only in neurons (which are derived from embryonic ectoderm); however, as outlined below, NF70 has now been found to exist also in chicken erythrocytes (which are derived from embryonic mesoderm). Chicken erythrocyte intermediate filaments are composed predominantly of a core vimentin polymer with the high molecular weight polypeptide synemin spaced at regular intervals along its length (Granger et al., 1982; Granger and Lazarides, 1982). Minor amounts of a 70,000 dalton (70 kd) polypeptide copurify with vimentin and synemin from these cells; this polypeptide comigrates with NF70 on two-dimensional (IEF/SDS) polyacrylamide gels, and has a two-dimensional chymotryptic iodopeptide map identical to that of NF70. Antibodies raised against chicken spinal cord NF70 crossreact specifically with the erythrocyte 70 kd polypeptide, as assayed by immunofluorescence microcopy and immunoelectron microscopy. Immunoautoradiography, Immunofluorescence microscopy and immunoelectron microscopy reveal that the erythrocyte 70 kd polypeptide is an integral component of erythrocyte intermediate filaments, and suggest that it copolymerizes randomly with vimentin to form core heteropolymers. The molar ratio of the 70 kd polypeptide to vimentin decreases during growth and development of the chicken as the erythrocyte population continuously turns over: on the average, the ratio is relatively high in embryonic erythroblasts and erythrocytes, less in erythrocytes from chicks and young chickens, and close to zero in adults. At all stages, there is a broad spectrum of ratios among different cells, but within individual cells the ratio does not seem to change significantly as the intermediate filament network is assembled. The 70 kd polypeptide is nearly absent from adult bone marrow erythroblasts and circulating erythroblasts from anemic adults, suggesting that expression of this polypeptide is a phenomenon related to the development of the organism rather than the differentiation of its erythrocytes. However, the wide range in the 70 kd polypeptide/vimentin ratio observed at any particular stage of development suggests that hematopoietic microenvironments or stem cell heritage may be important factors in the expression of the neurofilament polypeptide by erythrocytes. So far, no functional correlates of this expression have been elucidated, although it may be responsible for the observed change in the synemin periodicity during development (Granger and Lazarides, 1982). These findings also provide another example of unanticipated similarities between the nervous and hematopoietic systems; this is an intriguing but unexplained
phenomenon that has received considerable attention with respect to common cell surface antigens, but whose significance is unknown.

References:

129. SYNTHESIS AND ASSEMBLY OF SPECTRIN DURING AVIAN ERYTHROPOIESIS

Investigators: Ingrid Blikstad, W. James Nelson, Randall T. Moon

Similar to mammalian erythrocytes, avian erythrocytes have a submembranous spectrin-actin network and proteins analogous to mammalian ankyrin and the anion transporter. Previous studies have established that both embryonic and adult chicken erythrocytes contain two polypeptides (240,000 and 220,000 molecular weight, respectively), which by a number of criteria are analogous to mammalian erythrocyte α- and β-spectrin. We have examined the synthesis of α- and β-spectrin in erythroid cells at various stages of chick development and their assembly onto the membrane-cytoskeletal complex. Immunoprecipitations of Triton X-100-soluble and -insoluble cytoskeletal fractions with α- and β-spectrin antisera show that, at steady state, α- and β-spectrin are present in stoichiometric amounts and exclusively in the cytoskeleton. However, pulse labeling of cells and in vitro translation of total erythroid cell RNA reveal that α-spectrin is synthesized in a two to threefold excess over β-spectrin. Pulse-chase experiments show that newly synthesized α- and β-spectrin are present both in the soluble and cytoskeletal fractions, and that stoichiometric amounts are stably assembled in the cytoskeleton. On the other hand, there is a severalfold excess of α-spectrin relative to β-spectrin in the soluble fraction, both of which turn over with a half-life of ~50 min. In cells from 4-day-old embryos, more than 80% of the newly synthesized β-spectrin, but only 10% of the α-spectrin, are present in the cytoskeletal fraction. Thus, early in development, the association of α- and β-spectrin with the membrane-cytoskeleton may be rate limited by the amount of β-spectrin synthesized. Later on in erythroid development, progressively lesser proportions of newly synthesized β-spectrin are present in the cytoskeleton, suggesting that during development, the rate of association of β-spectrin with the membrane-cytoskeleton becomes limited by some other membrane cytoskeletal component.

130. IN VITRO SYNTHESIS OF ERYTHROID AND NONERYTHROID SPECTRIN AND THE REGULATION OF POSTTRANSLATIONAL ASSEMBLY

Investigator: Randall T. Moon

In erythrocytes, spectrin is the major component of the subcortical actin network or membrane skeleton. Spectrin, which is a dimeric protein consisting of nonidentical α and β subunits, binds to the inner surface of the plasma membrane by the noncovalent interaction of the β subunit with an extrinsic membrane protein, ankyrin. Recently, spectrin has also been found in nonerythroid cells (reviewed in Lazarides and Nelson, 1982), but the membrane receptor is unknown. Questions abound as to the pathways and mechanisms of assembly of the spectrin network in both erythroid and nonerythroid cells. It is difficult to study these processes in vivo because synthesis, assembly and turnover cannot be uncoupled readily. It would also be tedious to pursue in vitro experiments using classical biochemical approaches which would require initial purification of all of the nonerythroid variants of spectrin subunits. An in vitro system was therefore prepared from chicken embryo erythroid cells that is well suited for studying spectrin synthesis and assembly because the system synthesizes the spectrin subunits of interest. Looking first at erythroid spectrin, the in vitro system faithfully synthesizes full-sized erythroid α- and β-spectrin at the 3:1 ratio observed in vivo (Blikstad et al., 1983; see Abstract No. 129). Most importantly, the spectrin synthesized in vitro binds posttranslationally to both avian and mammalian erythroid cell membranes, and in the 1:1 stoichiometry observed in vivo. Under conditions that promote the binding to membranes of at least 95% of the available β-spectrin, any α-spectrin in excess of amounts equimolar to β-spectrin do not bind to membranes, suggesting that β-spectrin limits the binding of α-spectrin to the membrane. The rate of binding of β-spectrin to the membrane is itself limited by the
availability of membrane binding sites, as evidenced by the inefficient binding of β-spectrin to membranes not first depleted of endogenous spectrin. With regard to nonerythroid spectrins, the α, β and γ (a variant thought to be analogous in function to β-spectrin) subunits of lymphocyte and myoblast spectrins are currently being synthesized in a rabbit reticulocyte lysate programmed with mRNA from these sources and their assembly onto membranes is currently under investigation. By programming in vitro systems with mRNAs from divergent sources whose subunit composition of spectrin is known to be different, we can study the interaction of newly synthesized subunits with each other, their binding to both homologous and heterologous plasma membranes, and conversely, use labeled spectrins in vitro to search for non-erythroid spectrin membrane receptors. This approach, therefore, has great potential for studying the transition of multiple forms of newly synthesized structural proteins from the soluble phase to what may be a highly mosaic membrane skeleton.

References:

131. THE SITE OF SYNTHESIS OF α-SPECTRIN IN AVIAN ERYTHROID CELLS
Investigator: Ingrid Blikstad
The shape and structural integrity of the erythrocyte plasma membrane is maintained by a subcortical cytoskeletal network. Although the molecular organization of the mammalian erythrocyte plasma membrane is well understood, little is known about the site of synthesis and assembly of this membrane-cytoskeletal complex that occurs during erythropoiesis. In mammalian erythroid precursor cells, the membrane component of this complex, the anion transporter, is inserted cotranslationally in the endoplasmic reticulum membrane before being transported to the cell surface. However, the site of synthesis of spectrin, which plays a key role in the assembly of the membrane-associated cytoskeleton, is unknown. To investigate this we have chosen chicken embryo erythroid cells as a model system. After various periods of 35S-methionine incorporation, erythroid cells from 10-day-old chicken embryos were lysed in a Triton X-100-containing buffer and separated into a TX-100-soluble and -insoluble (cytoskeletal) fraction. Analysis of these two fractions by two-dimensional gel electrophoresis after a short pulse-labeling period, reveals that α-spectrin nascent polypeptides are present predominantly in the TX-100-insoluble fraction. These polypeptides can be immunoprecipitated with α-spectrin antisera and the 35S-methionine incorporated into them during a short pulse can be chased into mature α-spectrin molecules. The α-spectrin nascent polypeptide chains are quantitatively released from the TX-100 cytoskeleton by treatment of lysed cells with puromycin, suggesting that they themselves are not associated with the cytoskeleton. A fraction of the newly synthesized mature α-spectrin molecules is rapidly incorporated into the cytoskeleton as shown by the fact that they are not released by the puromycin treatment; the rest are recovered in the soluble fraction. These results suggest that α-spectrin is synthesized in association with the cytoskeleton during chicken erythropoiesis and assembles onto the membrane-cytoskeleton post-translationally.

132. EXPRESSION OF SPECTRIN IN NONERYTHROID CELLS
Investigator: W. James Nelson
Spectrin is a high molecular weight protein composed of two nonidentical subunits, termed α-spectrin (molecular weight 240,000) and β-spectrin (molecular weight 220,000). It has been extensively characterized in the mammalian erythrocyte where it has been shown to be the major component of the cytoskeleton and is involved in mediating linkage of actin to the plasma membrane. These interactions result in the formation of a dense cytoskeletal meshwork beneath the plasma membrane that confers structural support to the membrane and also restricts the mobility of proteins in the plane of the membrane (Branton et al., 1981). Most if not all nonerythroid cells also contain a membrane-associated cytoskeleton; however, the identity of proteins involved in a general mechanism of membrane-cytoskeleton linkage has not been
described. We have now shown that polypeptides functionally, structurally and antigenically related to erythrocyte spectrin are found in nonerythroid cells that may be suitable candidates for mediating membrane-cytoskeleton interaction in these cells (Lazarides and Nelson, 1982).

Recent results from this laboratory have shown that antibodies raised against the α subunit of chicken erythrocyte spectrin crossreact with antigenically related polypeptides in a wide variety of nonerythroid cells (Repasky et al., 1982). Subsequently we have found that, at least in chickens, α-spectrins from erythrocytes and nonerythroid cells (e.g., brain, lens, muscle) have identical two-dimensional chymotryptic peptide maps. We now have raised antibodies to the α subunit of chicken erythrocyte spectrin and found that, unlike α-spectrin, polypeptides antigenically related to β-spectrin have a relatively restricted distribution in nonerythroid cells (Nelson and Lazarides, 1983). By using indirect immunofluorescence microscopy, immunoprecipitation and immunon autoradiography, we have detected equivalent amounts of α- and β-analogues in adult cardiac and skeletal muscle cells and in some cells of the nervous system; other cell types appear to have little or no β-spectrin. Instead, these cells express another polypeptide structurally and functionally related to β-spectrin, termed γ-spectrin (molecular weight 235,000) that does not crossreact with either α- or β-spectrin antibodies and was shown to be similar to γ-spectrin from other nonerythroid cells. β-Spectrin antibodies did not crossreact with any lymphoid polypeptide by the criteria of immunoprecipitation and immunon autoradiography. The distribution of spectrin during cell-surface receptor capping was followed using indirect immunofluorescence microscopy and fluorescein-conjugated concanavalin A (Con A). At time zero both α-spectrin and the Con A receptors were distributed evenly at the plasma membrane; fluorescence was obtained with α-spectrin antibodies only following permeabilization of the cell, showing that the antigen was distributed on the cytoplasmic face of the plasma membrane. After about 15 min, the Con A receptors had formed patches. At the same time, the α-spectrin distribution had altered and was localized directly beneath each patch. After 45 min, the patches of Con A receptors had coalesced to form a single polar cap as had those of the α-spectrin (Nelson et al., 1983).

These results show that lymphocytes, which have a relatively "fluid" plasma membrane as shown by the mobility of cell-surface receptors in the plane of the membrane, express predominantly α-spectrin. Previous results have indicated that the actin-cytoskeleton is involved in the capping of cell-surface receptors. In view of the coordinate kinetics of patching and capping of Con A receptors

References:

133. INVOLVEMENT OF SPECTRIN IN CELL-SURFACE RECEPTOR CAPPING IN LYMPHOCYTES

Investigators: W. James Nelson, Camilo A. L. S. Colaco

The presence of polypeptides antigenically related to erythrocyte spectrin in human and mouse lymphocytes of B and T cell origin was determined by immunoprecipitation, immunon autoradiography and indirect immunofluorescence microscopy (Nelson et al., 1983). α-Spectrin antibodies specifically immunoprecipitated a high molecular weight polypeptide doublet from all lymphocytes examined. Immunon autoradiography of these immunoprecipitates showed that only the upper component of the doublet (molecular weight 240,000) crossreacted with α-spectrin antibodies. The lower molecular weight polypeptide (molecular weight 235,000) did not crossreact with either α- or β-spectrin antibodies and was shown to be similar to γ-spectrin from other nonerythroid cells. β-Spectrin antibodies did not crossreact with any lymphoid polypeptide by the criteria of immunoprecipitation and immunon autoradiography. The distribution of spectrin during cell-surface receptor capping was followed using immunofluorescence microscopy and fluorescein-conjugated concanavalin A (Con A). At time zero both α-spectrin and the Con A receptors were distributed evenly at the plasma membrane; fluorescence was obtained with α-spectrin antibodies only following permeabilization of the cell, showing that the antigen was distributed on the cytoplasmic face of the plasma membrane. After about 15 min, the Con A receptors had formed patches. At the same time, the α-spectrin distribution had altered and was localized directly beneath each patch. After 45 min, the patches of Con A receptors had coalesced to form a single polar cap as had those of the α-spectrin (Nelson et al., 1983).

These results show that lymphocytes, which have a relatively "fluid" plasma membrane as shown by the mobility of cell-surface receptors in the plane of the membrane, express predominantly α-spectrin. Previous results have indicated that the actin-cytoskeleton is involved in the capping of cell-surface receptors. In view of the coordinate kinetics of patching and capping of Con A receptors
and spectrin, and the emerging evidence that erythroid and nonerythroid spectrin are functionally and structurally related (Lazarides and Nelson, 1982), spectrin may be involved in mediating linkage of actin microfilaments to the plasma membrane and, indirectly, to the cell-surface receptors. We are currently investigating the protein-protein interactions involved.

References:

134. SWITCHING OF THE SUBUNIT COMPOSITION OF SPECTRIN DURING MYOGENESIS IN VITRO

Investigator: W. James Nelson

Avian myogenesis in vitro involves an initial phase of proliferation by primary chicken myoblasts before they undergo terminal differentiation. Differentiation is marked by the fusion of myoblasts to form multinucleated myotubes, the cessation of DNA replication, the synthesis of muscle-specific proteins and the elaboration of the contractile apparatus. To follow the expression of spectrin during myogenesis, cells at various stages of differentiation were pulse-labeled with $^{32}$S-methionine and the various spectrin polypeptides identified by immunoprecipitation using the α- and β-spectrin antibodies (Nelson and Lazarides, 1983). Initially, the unfused, rapidly dividing myoblasts express predominantly α-spectrin. An analysis of the rates of synthesis and turnover of these polypeptides indicates that the relative amount of α-spectrin in the cell remains constant during this phase of proliferation. Following fusion and during the subsequent phase of differentiation, the spectrin subunit composition of the myotube gradually switches to the αβ-spectrin phenotype. This switching is accomplished by a differential stabilization of γ-spectrin in myoblasts and then β-spectrin in the differentiating myotubes. During differentiation, the rate of turnover of spectrin decreases from ~14 hr in the myoblast (for αγ-spectrin) to >150 hr in 15-day myotubes (for αβ-spectrin). The gradual accumulation of αβ-spectrin during differentiation is also accompanied by the redistribution of spectrin from a diffuse membrane-associated meshwork in the myoblast to an intricate grid-like lattice in the well differentiated myotubes. In adult skeletal muscle, the αβ-spectrin complex is distributed on the sarcolemma as a ring or collar around each Z disc (Nelson and Lazarides, 1983), thus linking the Z-disc scaffold (Granger and Lazarides, 1979) to the sarcolemma.

The switching of the spectrin phenotype during myogenesis is correlated also with a gradual decrease in the "fluidity" of the plasma membrane, as shown by a decrease in the mobility of acetylcholine receptors, and a redistribution of the cytoskeleton. Previous studies from this laboratory have shown that intermediate filaments are distributed randomly in the cytoplasm of the myoblast, but become associated with the peripheries of the Z-disc in differentiating myotubes (Gard and Lazarides, 1980) to form an integrated Z-disc scaffold (Granger and Lazarides, 1979). Taken together these results show that during differentiation there is a change in the functional requirements of the cytoskeleton-plasma membrane complex. This change may be mediated by a switching of the subunit composition of spectrin, which may alter the dynamics of the interaction between the cytoskeleton and the plasma membrane.

References:

135. HIGHLY HOMOLOGOUS POLYPEPTIDES IN MUSCLE AND ELECTROLYTES FROM TORPEDO CALIFORNICA RELATED TO CHICKEN DESMIN

Investigator: W. James Nelson

The amino acid sequence of the intermediate filament proteins (IFP), vimentin and desmin, of
higher vertebrates demonstrates the homology between these two proteins (Geisler and Weber, 1982) and suggests that they have evolved from a common ancestral polypeptide. To investigate this we have characterized the intermediate filament protein(s) of Torpedo californica. Intermediate filament proteins were isolated by single-stranded DNA (ssDNA) cellulose chromatography (Nelson et al., 1982) of low ionic strength extracts of muscle and electrolytes, respectively. In each case a single polypeptide was isolated that had an identical molecular weight and isoelectric point to chicken desmin isolated from gizzard smooth muscle. The T. californica IFP had several physicochemical properties in common with higher vertebrate IFPs including similar solubility properties, affinity for ssDNA cellulose and susceptibility to degradation by a Ca$^{2+}$-activated proteinase (Nelson and Traub, 1982). Immunoblotting of the T. californica IFP from muscle or electrolytes reveals that it crossreacts with antibodies specific for chicken vimentin and desmin, respectively, suggesting that it is antigenically related to both chicken IFPs. To investigate this in more detail, we compared the two-dimensional chymotryptic peptide maps of chicken desmin and vimentin with those of T. californica muscle and electrolyte IFP. The results show that: 1) the T. californica muscle and electrolyte IFPs have identical peptide maps; 2) the T. californica IFP has extensive homology with chicken desmin; and 3) the T. californica IFP has several peptides (<15%) in common with chicken vimentin that are not common to chicken desmin.

References:

136. ADP-RIBOSYLATION OF A STRESS-INDUCIBLE PROTEIN IN AVIAN AND MAMMALIAN CELLS IN VIVO

Investigator: Lars Carlsson

ADP-ribosylation occurs as a posttranslational modification of a small group of proteins. The reaction is usually thought to be catalyzed and enhanced by cholera and diphtheria toxin, and in the cases examined, a specific enzymatic or regulatory function is known to be inhibited by the covalent attachment of ADP-ribose to the protein acceptors. Recently, several structural proteins have been shown to be modified by ADP-riboseylation, as well as a response to cholera or diphtheria toxins in vitro. What proteins function as cytoplasmic ADP-ribose acceptors in vivo is presently unknown.

ADP-ribosylation of proteins was analyzed by in vivo labeling of cells with $^{3}H$-adenosine, followed by separation of their protein components by two-dimensional isoelectric focusing/NaDodSO$_4$ polyacrylamide gel electrophoresis. In several cell types of avian and mammalian origin, the major $^{3}H$-adenosine acceptor in vivo (except for nucleic acids) is a polypeptide with a molecular weight of 83,000 and pl of 5.3. This polypeptide is identical to one of the stress-inducible and glucose-regulated proteins (SP 83), previously described in avian and mammalian cells. Snake venom phosphodiesterase digestion of purified $^{3}H$-labeled SP 83 releases 5'-AMP and a minor fraction of 2'-(5'-phosphoribosyl)-5'-AMP. In vitro labeling with $^{32}P$-NAD$^+$ of total cell lysates made in the presence of non-ionic detergents also results in incorporation of radioactivity into SP 83. Both of these results strongly suggest that the modification is an ADP-ribosylation. Heat shock and glucose starvation of cells induce a rapid and extensive decrease (ten and fivefold, respectively) in the incorporation of ADP-ribose into SP 83, suggesting that ADP-ribosylation may be important for the regulation of the function of this protein.

PUBLICATIONS


Professor: Jean-Paul Revel
Visiting Associates: Daniel B. Gros, Yves D. Plancke
Senior Research Fellow: S. Barbara Yancey
Research Fellows: Cheryl M. Corsaro, James D. Hatton
Graduate Student: Bruce J. Nicholson
Research Staff: James Dunn, Jean E. Edens, Les B. Grim, Patrick F. Koen
Support: The last year has proved very exciting because we are gradually getting a much clearer understanding of the organization of the gap junction protein in membranes. Bruce Nicholson and Daniel Gros have refined their analysis of heart inclusion...
junction protein and find it different from liver or lens when they are compared by peptide mapping; maps have been produced after proteolysis with several enzymes and after two different labeling techniques. Just how different the junction proteins from different tissues really are will only become clear, however, when complete sequences become available. Barbara Yancey is making very good progress towards the isolation of gap junction genes, using oligonucleotide probes synthesized by Dr. S. Horvath of Lee Hood's lab. The cloning is a collaborative effort with Dr. M. Gorin and Joe Horwitz of UCLA. As this goes to type we have in fact evidence that we have cloned the gene for the lens MP26, the protein believed to represent the junction protein in lens. Cheryl Corsaro's efforts at isolating a gap junction mutant continue. She has uncovered some very interesting purine metabolism mutants which should prove particularly useful in unraveling the mechanism of action of several important drugs. She has also been able to devise a new approach toward the production of gap junction mutants which we believe should be successful. All in all a very productive year, auguring well for the future.

137. FURTHER CHARACTERIZATION OF THE HEART GAP JUNCTION PROTEIN

Investigators: Bruce J. Nicholson, Daniel B. Gros, Jean-Paul Revel

Since the last report we have found a component of molecular weight (MW) 30,000 to be consistently associated with our heart gap junction fractions. Two-dimensional peptide mapping of peptides obtained after isolation of the proteins with or without protease inhibitors strongly suggests that the 30,000 kd protein is the "native" heart gap junction protein and is a precursor of the MW 28,000 protein which forms most of the heart gap junction fraction. The heart gap junction protein appears to be different from the proteins of liver and lens junctions as determined by a comparison of two-dimensional peptide maps. This has now been confirmed with both chymotryptic and tryptic two-dimensional maps of proteins iodinated by the chloramine T or Bolton and Hunter methods. A third dimension of resolution, achieved by running peptides eluted from the two-dimensional maps on high pressure liquid chromatography, failed to reveal any homologies between liver and heart gap junctional proteins. However, there was some suggestion of a possible conservation of the tertiary structure of these proteins reflected by similar protease sensitivities while part of the intact junctional structure. Trypsin treatment of intact heart junctions was found to cleave the heart MW 30,000 native protein into two MW 11,000 polypeptides, in a manner reminiscent of the formation of two tryptic MW 10,000 polypeptides from liver junctions (see Abstract No. 138). Further work is currently in progress to obtain sequence data on the heart gap junction protein to determine the actual degree of diversity between the structural proteins forming the gap junctions of different tissues.

138. FURTHER FRAGMENTS OF THE LIVER GAP JUNCTION PROTEIN FOR SEQUENCE ANALYSIS

Investigators: Bruce J. Nicholson, Jean-Paul Revel

Trypsinization of intact gap junctions cleaves the junctional protein of liver into two MW 10,000 fragments, one of which is the N-terminus of the "native" molecule. Since it is likely, from size considerations, that trypsin only hydrolyzes gap junctional protein exposed at the cytoplasmic faces of the junction, we can deduce that the N-terminus of the second MW 10,000 fragment must protrude into the cytoplasm before entering the membrane, which would protect it from further proteolysis. Given this information, it would be of great interest to sequence this portion of the junctional protein; however, it has proven very difficult to separate the two MW 10,000 fragments. The solution to this problem has been provided by the finding of an intermediate, stable stage in the chymotryptic hydrolysis of intact gap junctions. Here, the "native" molecule is cleaved into two fragments of MW 14,000 and 10,000, which are readily separable by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Peptide mapping has shown that the first of these represents the N-terminal half of the "native" protein, while the second corresponds to the other MW 10,000 trypsic polypeptide. It should now prove possible to sequence this new portion of the liver gap junction protein.
139. FURTHER SEQUENCING OF THE LENS MIP 26 JUNCTIONAL PROTEIN

Investigators: Larry J. Takemoto*, Bruce J. Nicholson, Michael W. Hunkapiller, Jean-Paul Revel

The main intrinsic protein (MIP 26) of eye lens fiber cell plasma membrane (Mr 26,000) has been associated with gap junction-like structures isolated from lens. The identity of these structures is the subject of widespread controversy, and the demonstration of any sequence homology between its major component and that of a gap junction in another tissue (e.g., liver) would help to settle the issue. Consequently we have been attempting to obtain further sequence data on both liver and lens junctional proteins. Cyanogen bromide digestion of isolated MIP 26 yields a major fragment of Mr 14,000 (termed CB-1) which, from carboxypeptidase digestions, has been demonstrated to represent the C-terminal half of the native protein. Since this represents an analogous location in the lens molecule to that occupied by the Mr 10,000 chymotryptic fragment in the liver gap junction protein (see Abstract No. 138), this provides an excellent opportunity for comparative sequence analysis beyond that already achieved at the N-termini of the native proteins (Nicholson, 1983). A sequence of about 30 residues has now been obtained for CB-1 from bovine lens. While the sequence is not particularly hydrophobic, a possible region which could be buried in the lipid bilayer has been identified. Comparisons with the corresponding liver sequence currently await sequence analysis of the chymotryptic Mr 10,000 fragment from the liver (see Abstract No. 138).

Reference:

*Department of Biology, Kansas State University, Manhattan, Kansas.

140. USE OF SYNTHETIC OLGONUCLEOTIDES TO CONSTRUCT AND SELECT cDNA FOR GAP JUNCTION PROTEIN

Investigators: S. Barbara Yancey, Michael B. Gorin*, Joseph Horwitz*, Jean-Paul Revel

The major protein component of gap junctions isolated from rat liver has been sequenced for 52 amino acids beginning at the NH₂-terminus (Nicholson et al., 1981). In order to pursue our goal to obtain a complete sequence of the protein, we have initiated experiments to obtain a sequence of its mRNA. Analysis of the available amino acid sequence data shows two regions where the ambiguity in the nucleotide coding sequences is minimal. Based on this analysis two mixtures of 14-base long oligonucleotides representing all possible coding sequences predicted for the amino acids have been synthesized by Dr. Suzanna Horvath (Dr. Hood's laboratory). Oligonucleotide I is a mixture of eight sequences complementary to the codons for amino acids 1 to 5, and oligonucleotide II is a mixture of 16 sequences complementary to the codons for amino acids 44 to 48. In order to construct a cDNA library enriched for sequences coding for the gap junction protein, we have used oligonucleotide II as a primer for reverse transcription of poly(A) RNA isolated from rat liver. To be assured of cloning the 5' sequences of mRNA which would be recognized by both oligonucleotides, dCMP tails were added to the 3' end of the first strand and oligo(dG) was used to prime the synthesis of the second strand. The double-stranded cDNA was tailed and cloned with pBR322 as the vector and E. coli MC 1061 as host. Initial screening of the cDNA library resulted in the isolation of a clone to which both probes hybridized but which, nevertheless, did not contain the expected sequence for the gap junction protein. By screening the library with the insert of this clone, we have eliminated it from the library and are presently rescreening for additional positive clones.

Reference:

*Jules Stein Eye Institute, University of California at Los Angeles.

141. STUDIES TO ISOLATE cDNA CLONES CODING FOR THE MAIN INTRINSIC PROTEIN OF BOVINE LENS

Investigators: S. Barbara Yancey, Michael B. Gorin*, Joseph Horwitz*, Jean-Paul Revel

From peptide mapping and partial amino acid sequences (Nicholson et al., 1983), no homology has as yet been detected between the liver gap junction
protein and the main intrinsic protein (MIP 26) of which the lens gap junctions are believed to be comprised. Sequences of the respective mRNAs could provide a more complete comparison to determine whether there is any homology between these two proteins, both of which are believed to mediate cell-cell communication. In order to characterize the mRNA for the MIP 26 we have constructed a cDNA library which we are screening with synthetic oligonucleotide probes. An amino acid sequence has recently been obtained for a cyanogen bromide fragment of MIP 26 from bovine lens (see Abstract No. 139). Nucleotide sequences complementary to all possible coding sequences for a stretch of seven of these amino acids have been deduced. From this information, 20-base long oligonucleotide probes have been synthesized by Dr. Suzanna Horvath (Dr. Hood's laboratory) as two mixtures, each consisting of 32 variations. The probes have been used to screen by colony hybridization a cDNA library constructed by reverse transcription of oligo(dT) primed poly(A) RNA isolated from bovine lens. The clones which were positive in the first screening were rescreeened and characterized with restriction endonuclease digestion. Sequencing is now in progress.

Reference:

*Jules Stein Eye Institute, University of California at Los Angeles.

142. THE USE OF CELL COMMUNICATION TO ISOLATE NOVEL MUTANTS IN PURINE METABOLISM

Investigators: Cheryl M. Corsaro, Mark L. Pearson*, Jean-Paul Revel

We have developed a selective method, based on the transfer of drug metabolites through gap junctions, for the isolation of mammalian cell mutants which are resistant to the metabolized form of a drug, rather than the drug itself. This involves the coculture of two cell types, one which can metabolize the drug and one which cannot. As a specific example, we cocultured hprt+ (hypoxanthine-guanine phosphoribosyltransferase) and hprt– rat L6 myoblasts in 6-thioguanine (6TG); the hprt+ cells metabolize 6TG into the toxic nucleotide 6-thioguanylate (6-TGRP) and transfer it to the hprt– cells through gap junctions. This selects for mutants of the hprt– cells which are resistant to the transferred 6-TGRP because of either an internal mutation in purine metabolism or a mutation in gap junctions (gap+). Up to now, all the mutant clones isolated have been gap+, as assayed by metabolic cooperation; thus we believe we have isolated purine metabolism mutants. One clone of these hprt–6-tgrpR mutants is being analyzed genetically, biochemically, and morphologically. hprt+ revertants of this clone were isolated and analyzed; the revertants have hprt activity equivalent to wild-type levels, but are still resistant to 6TG and are also resistant to 6-mercaptopurine, thus confirming that the 6-tgrp resistance is independent of the hprt deficiency. This clone is being assayed for alterations in enzymes thought to be regulated by 6-TGRP. It has also been shown to be gap+ by freeze-fracture electron microscopy and by the transfer of Lucifer yellow.

The significance of this mutant is that the novel way in which it was selected has potential for general use. This junction-dependent selection scheme could be used with other purine and pyrimidine analogues or other drugs (and the appropriately marked cells) to select a unique class of mutants which could be used to reveal the mechanism of action of the drugs used.

*NCI-Frederick Cancer Research Facility, Frederick, Maryland.

PUBLICATIONS


CELLULAR NEUROBIOLOGY

Mary B. Kennedy
Henry A. Lester
Felix Strumwasser
Summary: Processing of information in the mammalian brain is carried out by multineuronal "circuits" formed by arrays of neurons that activate or inhibit each other via specialized contacts called synapses. We are interested in the molecular structure, function, and regulation of these synapses.

Most synaptic terminals share certain common features. They are filled with clusters of vesicles that contain one or more types of chemical transmitter. When the neuron is electrically active, calcium ion flows into the terminal and catalyzes fusion of the vesicles with the plasma membrane resulting in the release of chemical transmitters into the extracellular space. The transmitters then bind to one or more types of protein receptors that are clustered in the membranes of postsynaptic neurons. This usually results in the opening of an ion channel that either depolarizes or hyperpolarizes the cell. Lying underneath the receptors, in the cytoplasm of the postsynaptic neuron, is a structure composed of protein filaments and referred to as the "postsynaptic density." The role of this structure is unknown, but it has been suggested that it may be involved in maintaining receptor clusters, and/or in mediating some of the physiological effects of receptor activation.

This simple picture of a synapse tends to obscure important aspects of the role of synapses in information processing. Physiological evidence suggests that the amount of transmitter released by each presynaptic impulse can be transiently turned up or down by a variety of mechanisms (Rosenthal, 1969; Mudge, 1979; Kandel and Schwartz, 1982). Some of these mechanisms appear to involve activation of receptors on the presynaptic terminals themselves. Others involve changes induced in the terminal by its own prior electrical activity. Similarly, relatively long-lasting transient changes in certain ion channels in the postsynaptic membrane have been shown to alter the excitability of neurons (Drummond et al., 1980; Kupfermann, 1979). This modulation of the magnitude of release of chemical transmitters, and of the ability of postsynaptic cells to respond to those transmitters, can alter the flow of information through neuronal circuits.

One way that neurons have been classified into types is according to the kinds of transmitters they release. It appears that neurons also differ in their regulatory machinery, that is, in the particular ways in which the strength of their synapses can be modulated. Consequently, a complete understanding of the functional organization of neuronal circuits requires information about not only the kinds of neurotransmitters released by each synapse but also the regulatory mechanisms present in each synapse. Characterization of the different forms of molecular machinery involved in modulation of synaptic efficacy will result in a clearer understanding of the workings of individual synapses, and will also allow immunocytochemical mapping, within complex brain regions, of sub-populations of neurons that contain particular modulatory mechanisms.

We are using biochemical and immunocytochemical techniques to characterize a synaptic regulatory protein that responds to changes in the intracellular concentration of calcium ion. Our approach is based on an analogy with the endocrine system. Many endocrine hormones exert their effects by raising the intracellular concentrations of "second messengers" such as cAMP or calcium ion (Robison et al., 1968; Rasmussen, 1977). These messengers, directly or indirectly, alter the activity of target cell proteins. The mechanisms by which this is accomplished often involve activation of protein kinases. A wealth of evidence now indicates that protein kinases are involved in the regulation of many important neuronal processes, including modulatory mechanisms such as those described above (Green, 1978, 1981; Kennedy, 1983). However, much remains to be learned about
the details of these mechanisms. We have previously described and partially characterized a calcium and calmodulin-activated protein kinase that is highly concentrated in brain (Kennedy and Greengard, 1981; Kennedy et al., 1983) and phosphorylates a synaptic vesicle-associated protein called synapsin I.

In the past year, we have purified the kinase, determined its subunit structure, and prepared a series of monoclonal antibodies that recognize it. In the course of this work, we have learned that the calmodulin-dependent synapsin I kinase is an unusually abundant brain enzyme, constituting as much as 0.3% of the total brain protein. This suggests that the kinase may be involved in the regulation of several neuronal processes and that activation of the kinase may be a major biochemical response of neuronal tissue to increases in calcium concentration. In keeping with this notion, we have found that the kinase phosphorylates brain proteins in addition to synapsin I. We have also obtained biochemical and immunochemical evidence that the kinase may be a prominent constituent of certain brain postsynaptic densities. Thus the kinase may be involved in the calcium-mediated regulation of postsynaptic as well as presynaptic events.

References:

143. PURIFICATION OF A RAT BRAIN CALMODULIN-DEPENDENT PROTEIN KINASE

Investigators: Mark K. Bennett, Ngozi E. Erondu, Mary B. Kennedy

We have completed the purification of a rat brain calmodulin-dependent protein kinase. The purification of the kinase was monitored by its ability to phosphorylate a synaptic vesicle-associated protein called synapsin I in the presence of calcium, calmodulin, magnesium and ATP. The purification procedure involved the following steps: 1) DEAE cellulose chromatography; 2) ammonium sulfate fractionation; 3) calmodulin-Sepharose affinity chromatography; 4) gel filtration chromatography; and 5) sucrose density gradient centrifugation. The enzyme is present in both soluble and particulate forms in fractions of brain homogenates. The particulate form is indistinguishable from the soluble form by several criteria (Kennedy et al., 1983). We have carried out the complete purification of the soluble form. The final product represents a 290-fold purification over the crude homogenate with 1.2% recovery of kinase activity from the homogenate, and 2.9% recovery from the soluble fraction. The recovery of purified protein kinase indicates that it is a relatively abundant brain enzyme comprising approximately 0.3% of the total brain protein. Its abundance in brain homogenates has been confirmed by quantitative immunochemical measurements.

The purified enzyme is composed of three subunits that comigrate with kinase activity during the purification steps and are coprecipitated with kinase activity by a specific antikinase monoclonal antibody. The three peptides have molecular weights of 50,000, 58,000, and 60,000 and have been termed α, β, and β', respectively. The α-subunit appears homogeneous and is immunologically distinct from the β-subunits. However, β' may have been generated from β by proteolysis. Sequence analysis will be necessary to determine the number of distinct β-subunits. Based on densitometric scans of Coomassie blue and fast green stained SDS/polyacrylamide gels, these peptides constitute 90-95% of the protein in the purified kinase. The only other distinct band on SDS gels of the purified kinase has a molecular weight of 45,000 and has been identified as actin. Because the amount of actin present varies from 2-5% and does not correlate with enzyme activity we have concluded that it is a persistent contaminant and is not a stoichiometric part of the holoenzyme complex.
However, we cannot rule out that the kinase has a specific but low affinity actin binding site.

References:

144. CHARACTERIZATION OF RAT BRAIN CALMODULIN-DEPENDENT PROTEIN KINASE

Investigators: Mark K. Bennett, Mary B. Kennedy

In order to define the subunit composition of the protein kinase holoenzyme, we investigated its hydrodynamic properties. The Stokes radius of the kinase was 95 Å, determined by gel filtration, and the sedimentation coefficient was 16.4 S, determined by sucrose density gradient centrifugation. A molecular weight for the kinase holoenzyme of approximately 650,000 was calculated from these values. As described in the previous abstract, the enzyme contains three subunits, α, β, and β'. We used densitometric scans of SDS/polyacrylamide gels stained with Coomassie blue or fast green to determine the relative amounts of each subunit in the purified enzyme. For this analysis we grouped the β-subunits and treated them as one. The subunits are present in a ratio of about three α-subunits to one β/β'-subunit. We postulate that the 650,000 dalton holoenzyme consists of approximately nine α-subunits and three β/β'-subunits.

The calmodulin-binding subunits of the kinase were identified by a calmodulin-SDS/polyacrylamide gel overlay technique (Carlin et al., 1981). 125I-calmodulin bound specifically to all three subunits of the enzyme. We have also found that the three subunits are autophosphorylated under conditions in which the kinase is active. The functional significance of this autophosphorylation is currently being investigated. This latter finding confirms the hypothesis suggested in earlier reports (Kennedy et al., 1983; Biology 1982, No. 147) that three prominent "substrate" proteins for a calmodulin-dependent protein kinase in brain homogenates are actually autophosphorylated subunits of the calmodulin-dependent protein kinase itself.

To facilitate comparison of the synapsin I kinase with other calmodulin-dependent kinase activities, we have investigated the ability of the purified kinase to phosphorylate kinase substrates other than synapsin I. Microtubule associated protein, smooth muscle myosin light chain (but not skeletal muscle myosin light chain), histone H3, and phosvitin are substrates for the enzyme. Phosphorylase b, glycogen synthase, and casein do not appear to be substrates. The nature of other apparent endogenous brain substrates is under investigation.

References:

145. SELECTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RAT BRAIN SYNAPSIN I KINASE

Investigators: Ngozi E. Erondu, Mary B. Kennedy, Valerie L. Krieger, Cathy B. Shapiro

Monoclonal antibodies can be used as biochemical reagents that bind to individual antigenic determinants with high specificity and high affinity. They are useful experimental tools for affinity purification of an antigen and of messenger RNA coding for the antigen, immunocytochemical localization of the antigen in tissue sections, quantitative radioimmunoassay of the antigen in crude mixtures, and structural comparisons of antigenically related molecules.

We have generated a panel of monoclonal antibodies directed against the calmodulin-dependent synapsin I kinase. We used as antigen protein kinase that had been partially purified through the calmodulin-Sepharose affinity chromatography step (Biology 1982, No. 149). To select hybridomas of interest, we developed three screening procedures. The first screen was a modification of conventional solid phase radioimmunoassay methods in which the hybridoma supernatants were tested for the presence of antibody recognizing any of the peptides in the crude antigen. We have selected about 80 clones that were positive in this assay. The second screen is a variation of the immunoblot method (also called "Western" blot) by which individual peptides recognized by each monoclonal antibody can
be identified. The third screen is a precipitation assay that tests for antibodies that bind to the synapsin I kinase in solution.

The last two screens are being used to classify the monoclonal antibodies secreted by hybridomas that were selected in the first screen. Several of the antibodies react with the kinase in solution and can precipitate kinase activity. These react weakly or not at all with the kinase subunits on immunoblots. Another class of antibody reacts well with one of the kinase subunits on immunoblots but does not bind to the kinase in solution. Two of the antibodies are specific for actin, another recognizes fodrin, a calmodulin-binding protein present in the crude antigen.

We have used one of the precipitating α-kinase monoclonals to confirm the subunit composition of the purified kinase (see Abstract No. 143). We are now developing radioimmunoassay techniques to study the distribution of the kinase in different brain regions, as well as in other tissues and species.

146. CALCULUM AND CALMODULIN-DEPENDENT PHOSPHORYLATION OF MICROTUBULE ASSOCIATED PROTEIN

Investigators: James M. Soha, Mary B. Kennedy

Microtubules, formed by polymerization of tubulin dimers, are abundant in neural tissue, occurring prominently in axoplasm, and in post-synaptic regions (Matus et al., 1981). High molecular weight (300,000 Da) microtubule associated proteins (MAPs) facilitate polymerization of tubulin dimers and are attached periodically to the assembled microtubules. One of these, MAP2, has been shown to be phosphorylated by cAMP-dependent protein kinase (Sloboda et al., 1975). This phosphorylation alters the effects of MAP2 on polymerization of microtubules and on crosslinking of actin filaments. Ca^{2+}-dependent phosphorylation of MAP2 has been observed previously in this laboratory in crude rat brain homogenates and in partially-purified microtubules. We have investigated the phosphorylation of MAP2 by purified calcium and calmodulin-dependent synapsin I kinase.

MAP2 is phosphorylated by the kinase at a high rate. The apparent K_m for MAP2 is 0.3 μM. The turnover number at saturation is about 2 moles of phosphate incorporated per second per mole of enzyme. Approximately 3 moles of phosphate are incorporated per mole of MAP2 when the reaction is allowed to run to completion, compared with about 2 moles of phosphate incorporated by the cAMP-dependent protein kinase.

In order to determine whether phosphorylation by the two kinases takes place at common or differing sites, we have compared two-dimensional tryptic phosphoprotein maps of MAP2 phosphorylated by each of the kinases. At least one site on the MAP2 molecule is specifically phosphorylated by the calmodulin-dependent protein kinase, and one or more additional sites are phosphorylated by the cAMP-dependent protein kinase. There also appears to be a single site that is phosphorylated by both kinases. Peptide maps of fragments generated by digestion of phosphorylated MAP2 with Staph. aureus V8 protease are consistent with this interpretation, as are the results of experiments in which MAP2 was phosphorylated sequentially by each kinase.

References:

147. IS THE CALMODULIN-DEPENDENT SYNAPSIN I KINASE INVOLVED IN GENERATION OF EPILEPTIC FOCI?

Investigators: Mary B. Kennedy, Claude Waularlain*

There are several animal models for the generation of epileptic foci. One of them is a phenomenon known as "kindling" (Racine, 1978). In the kindling paradigm, a chronic extracellular electrode is implanted in an appropriate region of a rat's brain. At regular intervals, separated by at least two or three hours and at most 24 hours, tiny electrical stimuli (400 μA, 1 sec) are delivered at the tip of the electrode. By the second or third stimulation, a short epileptiform afterdischarge can be recorded at the tip of the electrode following the 1-sec stimulus. The length of this afterdischarge increases with each stimulus until by about the twentieth stimulus, the animal experiences a full "stage five" seizure. This
effect is essentially permanent. After the regular stimulation is stopped for as long as several months, one or two stimuli will still generate a full seizure. Continual regular stimulation eventually results in spontaneous seizures. This effect is also permanent and the animal is said to have "kindled" epilepsy.

In recent years it has become clear that the "kindling" phenomenon results from a very long-term strengthening of synaptic connections along the stimulated neuronal pathways (Racine, 1978). In an effort to find biochemical changes that might underlie this change in brain function, Wasterlain and coworkers discovered a consistent decrease in calmodulin-dependent phosphorylation of certain proteins in the particulate fraction of homogenates of brain regions from kindled rats (Wasterlain and Farber, 1982). This change was correlated with the presence of kindling rather than with other variables such as implantation of electrodes or seizure activity itself. These proteins had the same molecular weights as the synapsin I kinase subunits. In collaboration with Claude Wasterlain, we have compared the proteins that change with kindling to the purified kinase subunits by one-dimensional SDS gel electrophoresis and by phosphopeptide mapping. By these criteria, the two sets of proteins are indistinguishable. The apparent change in the kinase subunits with kindling could have many different causes. The total amount of the kinase may have decreased. It may have moved from the particulate to the soluble fraction. The kinase may be more highly autophosphorylated in vivo, and thus take up less labeled phosphate in vitro. The brains of kindled animals may even contain more protein phosphatase activity. The biochemical tools that we have developed in the last year should allow us to distinguish among these possibilities. This experimental system may provide an opportunity to begin exploring specific physiological functions of the kinase.

References:

*Veterans Administration Medical Center, Department of Neurology, Sepulveda, California.

148. BIOCHEMICAL AND IMMUNOCHEMICAL EVIDENCE THAT THE α-SUBUNIT OF CALMODULIN-DEPENDENT SYNAPSIN I KINASE IS IDENTICAL TO THE "MAJOR 52K POSTSYNAPTIC DENSITY PROTEIN"

Investigators: Mary B. Kennedy, Mark K. Bennett, Ngozi E. Erondu

In an effort to understand the structure and function of brain postsynaptic densities (PSDs), several research groups have developed subcellular fractionation methods to isolate highly-enriched preparations of postsynaptic density-like material (Cotman et al., 1974; Cohen et al., 1977). These preparations have been reported to contain tubulin, actin, fodrin, calmodulin, and calmodulin-dependent phosphodiesterase and protein kinase activities. Whether these proteins are actually present in postsynaptic densities in vivo has not been confirmed.

A major constituent of PSD preparations from the cerebral cortex is a 52 kDalton protein that is distinct from both tubulin and various intermediate filament proteins (Kelly and Cotman, 1978). It has been shown to be a calmodulin-binding protein as well as a substrate for a calmodulin-dependent protein kinase that copurifies with the PSDs. Because of similarities in published characteristics of this protein and the α-subunit of calmodulin-dependent synapsin I kinase we have compared the two proteins directly. Rat brain synaptic plasma membrane and postsynaptic density nately density fractions were prepared by the method of Cotman et al. (1974). They were subjected to SDS gel electrophoresis in lanes adjacent to the purified synapsin I kinase. The "52K PSD protein" and the α-subunit comigrated on these gels. We used two methods to establish the chemical relationship between the two proteins. Immunoblots made with a monoclonal antibody that binds to the α-subunit revealed that the antibody binds equally well to the 52K PSD protein. Iodinated tryptic peptide maps of the two proteins were identical and were similar to those published by Kelly and Cotman (1978), who first described the 52K PSD protein.

Iodinated peptide maps of 60 kDalton proteins from SDS gels of PSD preparations revealed that the α-subunit of synapsin I kinase is also present in PSD preparations, although it is not the only protein present in this molecular weight region.
The α-subunit is 10-30% of the total protein in PSD preparations. If immunocytochemical experiments confirm its presence in PSDs in vivo, we can conclude that synapsin I kinase is likely to play a major role in the regulation of postsynaptic processes.

References:

**Professors:** Henry A. Lester, Jerome Pine*
**Visiting Associate:** Joël Nargeot
**Senior Research Fellow:** Jeanne M. Nerbonne
**Del E. Webb Research Fellows:** Lee D. Chabala, Alison M. Gurney
**Research Fellows:** Robert E. Sheridan Jr., Helen Rayburn*
**Graduate Students:** Mauri E. Krouse, John R. Gilbert*
**Research Staff:** John J. Beahan, Jr., Daniel R. Kegel, Roger Spencer, Theresa Stevens

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**Summary:** We are interested in exploring the mechanisms that render the nerve cell a highly sophisticated electrochemical machine, specialized to function on a time scale of milliseconds and a distance scale of micrometers.

In recent years the group has studied the synapse that transmits impulses from a nerve cell to a muscle fiber or (in certain fishes) to an electric organ. Acetylcholine is the transmitter at these synapses, and the group has explored the sequence of events that allows acetylcholine to interact with membrane-bound receptors and to open channels for ion flow in membranes. Several drugs and anesthetics can block this process, and the mechanisms of blockade provide important information about membrane function. The experiments utilize electrophysiological measurements—voltage-clamp and the like—in combination with a series of photoisomerizable molecules that resemble acetylcholine in some cases, and blocking drugs in other cases. Lasers and flashlamps are used to produce pulses of molecules near membranes, on the same time scale as the naturally occurring events; and the electrical measurements provide a simultaneous picture of how channels are responding in the membrane. This avenue of investigation seems to hold continued promise because of new techniques for recording single ionic channels in intact cells and in isolated membrane patches. We have adopted these "patch-clamp" procedures, using cultured cells. In our present experiments, photochemical manipulations are timed, by on-line data processing, to occur while a single channel is either open, closed, or desensitized.

The group is also investigating the processes that enable intracellular messengers—cyclic nucleotides, calcium ions and protons—to control ion channels and gap junctions in membranes. The rationale of these experiments is to produce photochemical "concentration jumps" of second messengers within intact cells under electrophysiological investigation. The work required the development of a new photochemical technology, based largely on photolyzable ortho-nitrobenzyl esters, and the

**Publications**
introduction of several new preparations in the laboratory. We have employed photochemical pH-jumps to turn off gap junction channels in Chironomus salivary glands. Also, the physiologically crucial Ca conductance of heart muscle is increased within a fraction of a second after an intracellular concentration jump of cyclic AMP—but not of cyclic GMP! We are looking forward to detailed information about the kinetics and localization of second messenger action.

Dr. Jerome Pine, Professor of Physics at Caltech, is affiliated with the research group. Pine's interests center on nerves and muscles in culture, and on those factors that influence synapse formation, receptor production, and synaptic change. Tissue culture dishes are used that incorporate microelectrode arrays, for long-term stimulation and recording. Other experiments involve the use of voltage-sensitive dyes to monitor electrical activity. An ultrasoft X-ray scanning microscope is being developed, with the goal of studying live, developing synapses with submicron resolution.

149. THE MEASUREMENT OF THE BINDING AND UNBINDING RATE CONSTANTS OF A COMPETITIVE ANTAGONIST

Investigators: Mauri E. Krouse, Henry A. Lester

Agonists and competitive antagonists are thought to bind to the same site on the nicotinic acetylcholine receptor. Antagonist binding to the receptor does not lead to channel activation, as does agonist binding, but instead inhibits agonist binding. Antagonist binding rates cannot be measured by voltage-jump relaxation experiments. The present experiments, using the light flash technique, allow us to monitor these rates for the first time.

The experiments use a single voltage-clamped cell from the electric eel, Electrophorus electricus. The voltage-clamp current is proportional to the number of channels opened by the agonist. When the cell is exposed to both a photostable agonist and a photoisomerizable antagonist, a voltage jump or light flash produces a relaxation to a new steady state level of agonist-induced current. An experimental trial comprises three episodes. During the first episode, voltage is jumped from +60 mV to -150 mV. During the second episode, the voltage jump is repeated; but in addition, after the current reaches equilibrium, a light flash occurs. During the third episode, only the voltage jump occurs. The agonist-induced conductance is increased by the flash and is the same at the end of the second and third episodes.

A flash-induced "down-jump" in antagonist concentration causes an increase in agonist-induced current with an exponential rate constant close to that of the subsequent voltage-jump relaxation (both relaxations involve channel activation). An "up-jump" in antagonist concentration causes a decrease in the number of open channels. This relaxation is not a single exponential; it has a sigmoidal waveform. The delayed start arises because agonist molecules must dissociate from the receptors before antagonist molecules can bind. When the agonist is carbachol, the light-flash relaxation is at least four times slower than the subsequent voltage jump relaxation. These antagonist kinetics imply a binding rate constant of 5x10^7 M^-1 sec^-1, roughly equal to that estimated for the more slowly-binding agonists. We are presently investigating the effect of temperature, voltage, and different agonists on the shape of the flash-induced relaxations.

150. PATCH-CLAMP RECORDINGS FROM SINGLE NICOTINIC ACETYLCHOLINE OIANNELS ACTIVATED BY A PHOTOISOMERIZABLE AGONIST

Investigators: Lee D. Chabala, Alison M. Gurney, Robert E. Sheridan, Henry A. Lester

We are studying the stochastic properties of single-channel currents from nicotinic acetylcholine (ACh) receptors in several different cultured muscle preparations. The cis isomer of the Bis-Q molecule is only a weak agonist for nicotinic ACh receptors, whereas the trans isomer is a powerful agonist and opens nicotinic ACh channels in cultured muscle preparations. Depending on the experimental conditions, a 1 ms light flash produces cis + trans or trans + cis photoisomerizations of the Bis-Q molecule; thus a photochemical manipulation produces a rapid change in agonist concentration near the ACh channel or it
perturbs the agonist-receptor complex. According to present concepts, the effective channel opening rate depends on agonist concentration and not on membrane potential. We are obtaining kinetic information by performing such manipulations while an individual channel is in a known initial state (i.e., open or closed).

Single-channel and whole-cell recordings are being carried out in conjunction with pharmacological light flash manipulations to study the agonist-receptor interaction. Experiments with pure cis-Bis-Q in the patch pipette indicate that, following a light flash to produce some trans-Bis-Q molecules, ACh channels begin to open. The distribution of waiting times to the first opening is being investigated, and a binomial analysis is being used to estimate the number of channels in a patch. Other experiments, in which a light is flashed while a channel is open, suggest that a flash promotes trans → cis photoisomerizations of some Bis-Q molecules bound to receptors. These channels close as either of the bound agonist molecules leave the appropriate receptor site. We are interested in the distribution of closing times as well as the distribution of renewal times for the channels to reopen. The single-channel measurements are also being used to help understand the whole-cell macroscopic membrane currents. Finally, we are interested in determining the dose-response relationship in terms of the relative frequency of opening of single channels as a function of agonist concentration.

151. **TIME COURSE OF THE INCREASE IN THE MYOCARDIAL SLOW INWARD CURRENT AFTER A PHOTOCHEMICALLY GENERATED CONCENTRATION JUMP OF INTRACELLULAR cAMP**

**Investigators:** Joëlle Nargeot, Jeanne M. Nerbonne, Joachim Engels*, Henry A. Lester

In this study, we established the usefulness of the light-flash technique for investigations on the control of channel properties by intracellular second messengers. The experiments exploited the properties of o-nitrobenzyl-cAMP and its cGMP analogue. These molecules are members of a group of phosphotriesters which enter cells. The o-nitrobenzyl esters can then be photolyzed to produce the cyclic nucleotides themselves, presumably within a few msec. Voltage-clamped atrial trabeculae from bullfrog hearts were exposed to o-nitrobenzyl cAMP or cGMP. Ultraviolet flashes produced intracellular concentration jumps of cAMP and cGMP. With the cAMP derivative, estimated intracellular jumps of 0.1 to 1 µM resulted in up to a fivefold increase in the slow inward current \( I_{sl} \). This increase led to a broadened action potential. \( I_{sl} \) reached a maximum 10-30 sec after the flash and declined over the next 60-300 sec. The first increases were observable within 150 msec; this value is an upper limit imposed by the instrumentation and contrasts with a delay of about 5 sec after the application of β-adrenergic agonists. Responses to flashes lasted longer at higher drug concentrations, and in the presence of the phosphodiesterase inhibitor, papaverine; effects of flashes developed and declined faster at higher temperature. Although the amplitude of \( I_{sl} \) was increased, its waveform and voltage-sensitivity were not affected. Intracellular concentration jumps of cAMP failed to affect the muscarinic K+ conductance. There were no observable effects of cGMP concentration jumps (up to 15 µM). The data confirmed (a) that cAMP regulated \( I_{sl} \) and (b) that the 5-10 second delay, observed in previous studies between application of β-agonists and the onset of positive inotropic effects, has been correctly ascribed to activation of adenylate cyclase.

We have recently synthesized and characterized an analogue of o-nitrobenzyl cAMP with methoxy groups on the 4 and 5 positions of the benzene ring. This modification shifts the absorption maximum about 90 nm toward the red, to 350 nm, which allows much greater photolysis with flashlamps, obviates the need for quartz optics, and minimizes tissue damage. This improvement should enable flash-activated cAMP jumps for many cells. In further experiments with the voltage-clamped bullfrog atrium, we find that the 4,5-dimethoxy compound is several times more potent than the original o-nitrobenzyl cAMP in stimulating \( I_{sl} \). We are presently using this molecule and its cGMP analogue to probe further the concentration dependence and the kinetics of the response to cyclic nucleotide concentration jumps.

*Fachbereich Chemie der Universität Konstanz.*
152. TENSION MEASUREMENTS ON BULLFROG ATRIAL MUSCLE DURING INTRACELLULAR CONCENTRATION JUMPS OF cAMP

Investigators: Joël Nargeot, Didier Garnier*, Sylvain Richard*, Jeanne M. Nerbonne, Henry A. Lester

These measurements, employing the double sucrose-gap technique, extended those described above on increases in the slow inward current, I_{si}, during photochemically induced concentration jumps of intracellular cAMP. Two types of contraction can be distinguished in frog heart. The phasic contraction produced by a brief depolarization is thought to depend on I_{si}, the influx of Ca^{2+} ions. A slower tonic contraction can be elicited by a long depolarization, even when I_{si} and the phasic contraction are inhibited by Mn^{2+} or Cd^{2+}. The tonic contraction is probably caused by a voltage-dependent liberation of Ca^{2+} from intracellular stores.

In the presence of o-nitrobenzyl cAMP (50 to 100 µM), a single UV flash caused a roughly twofold increase in the phasic tension over a time course of 30 sec; this agreed well with the magnitude and time course of the simultaneously measured increase in I_{si}.

It was also of interest to determine whether intracellular concentration jumps of cAMP could mimic the relaxing and positive chronotropic effects of β-adrenergic stimulation often seen in mammalian hearts. The tonic contraction was indeed decreased by cAMP concentration jumps, possibly because of increased Ca^{2+} pumping by the newly phosphorylated sarcoplasmic reticulum. In sinus venosus fibers stimulated to fire repetitively by depolarizing currents, the contraction and frequency were both increased by cAMP jumps. The latter effect may arise from the augmentation in the delayed potassium conductance produced by the cAMP concentration jumps; this augmentation in turn may be linked to the increased Ca^{2+} influx.

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153. WHOLE-CELL RECORDING OF CALCIUM CURRENTS INDUCED BY INTRACELLULAR CYCLIC NUCLEOTIDES IN ISOLATED ADULT HEART CELLS

Investigators: Alison M. Gurney, Jeanne M. Nerbonne, Roger W. Spencer

In order to extend the studies described above, we are studying isolated heart cells that allow more precise electrophysiological measurements. Individual ventricular cells are isolated from adult guinea-pig hearts by retrograde perfusion through the aorta with a mixture of collagenase and hyaluronidase in a Ca^{2+}-free Tyrode solution. Viable cells obtained by this procedure are relaxed and have a rod-like shape and striations. We plan to study changes in ion channel gating that depend on intracellular cAMP-stimulated protein phosphorylation. Whole-cell currents will be recorded in these cells while flashes are used to produce "concentration-jumps" of cyclic nucleotides. Together with patch-clamp studies of currents through single ion channels in electrically isolated patches of cell membrane, these experiments should provide information on the kinetics and localization of the second messenger action of the cyclic nucleotides.

154. THE CONTROL OF CALCIUM CHANNELS IN INTRACELLULAR SECOND MESSENGERS IN CULTURED CARDIAC MYOCYTES

Investigators: Alison M. Gurney, Lee D. Chabala

Cardiac calcium channels are gated by membrane potential and are regulated by intracellular second messengers such as cAMP. We are combining whole-cell and single-channel recording techniques with pharmacological manipulations to study the regulation of calcium channels in cultured myocytes from neonatal rat hearts. During a depolarizing step in membrane potential, the cardiac calcium channels open, then inactivate. When barium ions carry the inward current, the rate of inactivation is slower and the single channel currents are larger than when the current is carried by calcium; thus the barium ion is used as the charge carrier in our patch-clamp studies of the calcium channel. Only a minority of patches show calcium channels; hence we are trying to increase the density by supplementing the culture medium with prostaglandin E_{1}, which stimulates adenylate cyclase.

155. THE DESIGN OF PHOTOACTIVATED MOLECULES: CHEMICAL AND STRUCTURAL CONTROL OF REACTION RATES AND EFFICIENCIES

Investigator: Jeanne M. Nerbonne

Light-sensitive moieties have been widely used in organic synthesis. In an extension of this
approach, the α-nitrobenzyl functional group, in particular, has been exploited by several groups for the preparation of biologically useful photoactivatable compounds. Little is known, however, of the influence of the chromophore or the leaving group on the reaction rates and efficiencies. To probe these questions, we prepared a series of 12 photolabile proton donors with various chromophores, protecting groups and leaving groups. We find that the reaction rates depend on the nature of the leaving group and on the structure of the chromophore. Simple substitution on the aromatic nucleus increases the reaction rate ten to one hundredfold. All reactions show pH dependence, increasing two to threefold in rate for a decrease of one pH unit. We have also prepared analogous cAMP derivatives, and again find that the reaction rate can be increased one hundredfold by substitution on the aromatic nucleus of the protecting group. The reaction quantum yields, on the other hand, are not highly sensitive to any of the structural modifications made. All derivatives lead to efficient photorelease (quantum yields 0.4-0.9).

Each of these factors sheds light on the problem of designing photoactivated protecting groups that are fast, efficient and insensitive to the nature of the leaving group and, therefore, generally useful.

156. INTRACELLULAR pH AND GAP JUNCTIONAL PERMEABILITY IN EARLY EMBRYOS

Investigators: Jeanne M. Nerbonne

In collaboration with A. Harris, D. Spray, and M. V. L. Bennett (Albert Einstein College of Medicine), we have attempted to utilize the ortho-nitrobenzyl acetates to study the effect of intracellular pH on gap junctional conductance in developing blastomeres from squid, fundulus, and axolotl embryos. In all of these preparations, we find that exposure to the drugs causes a precipitous drop in junctional conductance and a large drop in intracellular pH in the absence of light (these compounds were originally designed as "light-activated" proton donors). Bath application of 1-2 mM of the ester rapidly (<1 min) reduces junctional conductance and recovery occurs in several minutes. Ester treatment decreases intracellular pH monitored spectrophotometrically with phenol red and/or with a pH microelectrode. Decreases in junctional conductance were reversed by NH₄Cl. Hydrolysis of the ester is negligible in physiological saline and, therefore, it appears that protons are generated by enzymatic cleavage of the ester bond. Esterase inhibitors, with some variability, prevent this reaction. Because it is difficult to inhibit hydrolysis completely and reproducibly, we have prepared and characterized two new classes of photosensitive proton donors. We are presently utilizing these compounds in several gap junction preparations.

157. PHOTOSENSITIVE CALCIUM CHELATES

Investigators: Jeanne M. Nerbonne, Donald C.-T. Lo

In many cells, alterations in intracellular calcium concentration interact with intracellular pH; furthermore, changes in internal calcium or pH have diverse physiological effects. To study these phenomena, we have been developing molecules that liberate calcium ions rapidly and efficiently upon irradiation. These compounds will be used alone or in conjunction with the photactivated proton donors to examine the intracellular buffering mechanisms for these small ions (on a wide range of time scales) and to measure directly the physiological responses to step changes of concentration.

We have taken two approaches to developing photosensitive calcium chelates. In the first case, we have exploited the well-characterized azobenzene photochemistry to prepare molecules with appropriately disposed carboxylate moieties. Depending on the wavelength of irradiation, these molecules can be photoisomerized reversibly between two states with different binding affinities. For the second class of compounds, irradiation produces an irreversible reaction that results in a large drop in the ligand affinity; free calcium is liberated. Several compounds of both classes have been prepared and their calcium and magnesium affinities determined using spectroscopic and electrochemical techniques. None of the compounds prepared to date binds calcium strongly enough (pCa 7-8) to be useful in intracellular studies. The
information gleaned from these studies has provided us with a definition of the structural and chemical requirements for optimization of calcium binding and we are hopeful that compounds with the requisite affinity can be prepared.

In a collaborative effort with J. Kaplan (University of Pennsylvania), we have begun synthetic work on another class of photosensitive calcium chelates: molecules that can bind calcium in the physiological concentration range only after the photoreaction.

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158. SYNAPSE DEVELOPMENT AND PLASTICITY IN CULTURE
Investigators: Jerome Pine, John R. Gilbert, Helen Rayburn

Culture dishes have been made which embody an array of 61 extracellular microelectrodes formed by a pattern of thin gold conductors on the dish surface. The electrodes are located in a regular array, spaced 70 microns apart, covering an area approximately 0.5 mm in diameter at the center of the dishes. Cultures of chick ciliary neurons and of rat superior cervical ganglion neurons are being grown in the dishes, and conditions for good growth and survival are being optimized. Also, various techniques for confining a few neurons to a microculture covering the electrode area are being evaluated.

Within a short time, we expect to begin studies of the connectivity of small neural networks formed by the cells of the microcultures, and of the influence of various factors, such as controlled chronic stimulation, on the synapses.

We are continuing our efforts to combine the use of voltage-sensitive fluorescent dyes with the microcircuit petri dishes. Much of the necessary apparatus has been constructed, and a collaboration with Amiram Grinvald's group at the Weizmann Institute has been initiated. We expect to begin experiments during the coming year.

During the past year we have refined the engineering of the microcircuit dishes so that they are easy to clean and reuse. Also, we are now completing the first prototypes of computerized stimulating and recording electronics.

159. SOFT X-RAY MICROSCOPY
Investigators: Jerome Pine, Jeanne M. Nerbonne, Helen Rayburn

The collaborative effort between ourselves and groups at the State University of New York and the IBM Watson Laboratories is aimed at the first explorations of ultrasoft X-ray microscopy of live wet cells. The microscope is now installed on a dedicated beam line at the National Synchrotron Light Source, at Brookhaven Laboratory. A focusing zone plate has been successfully used to produce a beam spot approximately 0.2 microns in diameter, which will make possible scanning microscopy of whole cells with that resolution. Since this is an initial effort, it is likely that improvement in the resolution by at least a factor of two will be forthcoming in the near future.

Our interest is in applying the microscope to cells grown in culture, and we have succeeded in growing networks of neurons on substrates 0.1 microns thick for the initial tests of the instrument. At this time, instrumental tests of the microscope are being concluded, and initial results from our cultures will be obtained in the coming months. At present the exposure time for a picture is of the order of one hour, but a new, more intense beam has been designed, and is planned to be completed during the next year. The intensity is expected to increase by a factor of at least 10,000, which will make the time required per picture negligible.

Although the resolution that the microscope will attain is relatively easy to predict, much of its usefulness will depend on the subtle ways in which the absorption of the X-rays generates contrast in wet, unstained material. We have verified that muscle cultures are seen with high contrast at the most convenient operating wavelength of 3.1 nanometers, in pictures taken during the past year, before the zone plate was completed. A one-micron-diameter pinhole was used in lieu of the zone plate. Much of the initial research during the coming year will be to investigate the contrast available at various wavelengths, and to quantify the expected ability of the microscope to perform elemental microanalysis as well as revealing ultrastructure.
PUBLICATIONS


Professor: Felix Strumwasser
Visiting Associate: John C. Woolum
Research Fellow: Karen Goldman Herman
Research Staff: Peter S. Campbell, Susan L. Mailheau, Laurie S. Minamide, Gloria I. Sanchez, Floyd Schlechte, John M. Scotese

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Summary: It is now known that peptides mediate important behavioral events in most nervous systems. The most complete details of a peptide-mediated behavior, egg laying, have been worked out in the mollusc Aplysia. This is primarily due to the accessibility of the bag cell (BC) neurons for studies at the physiological, biochemical and genomic levels. Lois Toevs, my first graduate student at Caltech, determined that the egg-laying hormone (ELH) extracted from the bag cell neurons was a peptide with molecular weight of ~6000 daltons. Arlene Chiu, for her doctoral research, purified ELH and raised antibodies to it with which she proved, by immunochemistry, that ELH was uniquely contained in the BC neurons among the cells of the abdominal ganglion. Eri Heller, a senior research fellow, purified two additional peptides, peptides A and B, from the atrial gland of the reproductive tract and Leonard Kaczmarek and I showed that these peptides caused the bag cells to discharge and thereby release ELH. Mike Hunkapiller, while in Lee Hood's lab, sequenced these three peptides. Finally, Richard Scheller, while in Richard Axel's lab at Columbia University, cloned and sequenced the genes for these three peptides and confirmed the peptide sequences determined here at Caltech. These studies, initiated in 1967 at Caltech, have demonstrated that it is possible to proceed from a known behavior to genes providing that peptide products can first be purified and assigned physiological roles in that behavior. Similar approaches should be possible to understand the circadian oscillator system situated in the eye of Aplysia which governs the cycle of daily locomotion.

160. INDIRECT DETERMINATION OF THE GENE EXPRESSION FOR PEPTIDES A AND B IN APLYSIA

Investigators: Laurie S. Minamide, Felix Strumwasser

It has been postulated by Heller et al. (1980) that during copulation, the atrial gland of Aplysia californica releases peptides (A and B) which act on neuronal receptors causing the peptidergic bag
cells in the abdominal ganglion to afterdischarge. In turn, the stimulated bag cells initiate egg laying through secretion of egg-laying hormone (ELH). Recent experiments have shown binding of independent rabbit polyclonal antibodies made to ELH and peptide B to proteins contained in bag cell homogenates which have been separated on 13.5% polyacrylamide gels and then electrophoretically transferred to nitrocellulose. The antigen-antibody complexes are identified by reaction with iodinated protein A. Interestingly, the antibody to ELH binds to two protein bands of which one appears to correspond to the known ELH precursor of molecular weight 41,800 daltons. The ELH antibody also binds heavily to a range of low molecular weight material below 6000 daltons, presumably ELH-like peptides. Similarly, the antibody to peptide B binds to two protein bands whose molecular weights correspond to those of precursors to both peptides A and B, 20,600 daltons and 13,800 daltons, respectively. However, there is no binding of the peptide B antibody to the same range of low molecular weight material antigenic to the ELH antibody. Because the molecular weight of peptide B is approximately 4000 daltons, it is suggested that although the genes for peptides A and B are expressed in bag cells, the final processing of peptide B to its bioactive form is not occurring as it is for ELH. Further experimentation will entail applying the ELH and peptide B antibodies to proteins separated from atrial gland homogenates to detect the presence of peptide B and its precursors and the possible absence of the ELH precursor and the low molecular weight material antigenic to the ELH antibody. (We thank Arlene Chiu for the ELH antibody and Joanne Yeakley and Arthur Roach for the peptide B antibody.)

Reference:

161. STRUCTURE OF cDNA CLONES FOR PRECURSORS TO NEUROACTIVE PEPTIDES FROM THE ATRIAL GLAND OF THE MOLLUSC APLYSIA


Egg laying in Aplysia is controlled by a neuropeptide, egg-laying hormone (ELH), synthesized by the bag cell (BC) neurons of the abdominal ganglion. The BC neurons in turn are activated by at least two peptides (peptide A and peptide B, molecular weights 3924 and 4032, respectively) synthesized in the atrial gland of the reproductive tract. Recently, three ELH-coding genes have been cloned and sequenced by Scheller et al. (1983). Our studies are directed at ultimately determining how the multiple genes in this small family are differentially expressed. We have constructed a cDNA library from poly(A)+ RNA isolated from the atrial gland. The library was screened using a DNA fragment from a genomic clone (ELH-1) coding for atrial gland peptide B precursor (Richard Scheller, Stanford University). Hybridization-positive clones constituted 5% of the total library, indicating that the precursor protein mRNA is highly represented in the atrial gland. DNA sequencing of two of the largest positive clones has almost been completed. One of these clones (S40) carries sequences almost identical to those reported as nucleotides 1293 to 1772 of the A-precursor by Scheller et al. (1983). These nucleotides encode the C-terminus of peptide A (residues 12-34) as well as a peptide with homology to ELH. However, our sequence differs from the published A-precursor sequence by three base substitutions and one deletion, all outside the coding region for the A-peptide.

References:

*Undergraduate, California Institute of Technology.

162. MONOCLOAL ANTIBODIES TO THE EYE AND BAG CELLS OF APLYSIA

Investigators: Laurie S. Minamide, John M. Scotese, Felix Strumwasser

Hybridoma technology has been applied in the hope of producing highly specific reagents for probing physiological function and protein distribution in the various cell types of the eye and peptidergic bag cell clusters of Aplysia californica. Of particular interest is the characterization and perhaps the determination of the cellular origin of the circadian oscillator system contained in the Aplysia eye. Additionally,
we plan to use the antibodies as probes to specifically interfere with physiological function, for example the afterdischarge in bag cells. Thus far, mouse monoclonal antibodies have been produced and are being screened by two methods: against antigenic preparations using nitrocellulose as a solid support, and immunocytochemically against fixed primary cell cultures. Production of ascites fluid has been used to generate higher antibody titers than in hybridoma cell culture, with subsequent purification of the immunoglobulins using DEAE Affi-Gel Blue and CM-Affi-Gel Blue from BioRad.

The first hybridoma IgG purified was produced from the fusion of NS1 myeloma and spleen cells from a bag cell immunized BALB/c mouse and has been shown to bind to four protein bands from a bag cell homogenate first separated electrophoretically on a 13.5% polyacrylamide gel and then transferred electrophoretically to nitrocellulose. The specificity of this antibody is obvious in comparison to the total number of protein bands, numbering more than 40, from bag cell homogenates separated on polyacrylamide gels and detected by Coomassie blue staining. As characterization of the different antibodies progresses, it is hoped that their specificity can be manipulated to further our understanding of the neurophysiology of Aplysia.

163. CALCIUM MEASUREMENTS IN BAG CELLS

Investigators: John C. Woolum, Felix Strumwasser

We have continued our measurements of dynamic changes in internal calcium ([Ca\(^{2+}\)]\(_i\)) in bag cells using the dye arsenazo III. Isolated bag cells in culture containing arsenazo III can be made to afterdischarge with N\(^6\)-n-butyl 8-benzyl thio cAMP. The much lengthened single action potentials (either stimulated or spontaneous) give rise to [Ca\(^{2+}\)] increases that are easily measured with the arsenazo system. Successive closely spaced action potentials show potentiation in [Ca\(^{2+}\)] increase. Passage of a long depolarizing current pulse produces a continuous rise in [Ca\(^{2+}\)] indicating that the calcium channels do not inactivate in times of tens of seconds. The small membrane potential oscillations present during an afterdischarge and the long, large regenerative hyperpolarizations sometimes present near the end of an afterdischarge are not associated with measurable changes in [Ca\(^{2+}\)]. The increase in [Ca\(^{2+}\)] associated with action potentials is quickly buffered (in 10 to 60 sec) by the cell so no large increase in [Ca\(^{2+}\)] occurs during an afterdischarge.

164. \(^{35}S\)-METHIONINE INCORPORATION INTO PROTEINS IN THE EYE OF APYLSIA DURING CIRCADIAN ACTIVITY

Investigators: Felix Strumwasser, George C. Stone*, Laurie S. Minamide

We have examined the pattern of proteins synthesized in the isolated eye of Aplysia at two different phase points in the circadian cycle of compound action potential frequency. Aplysia were entrained on 12 hour:12 hour white light:dim red light cycles in one of two constant temperature cubicles which differed only in the time of light onset and offset. Eyes with long optic nerves were always dissected during the dim red cycle and incubated in the dark for 2 hours in 350 µCuries/ml of \(^{35}S\)-methionine in Hepes buffered (pH 7.8) artificial sea water. At the end of incubation, the eyes were rinsed three times, the optic nerves were removed and four eyes were homogenized in an SDS-NP40-urea solution containing pH 4-10 ampholytes (Ames and Nikaido, 1976; Wilson et al., 1977). Isoelectric focusing provided a separation of proteins, according to charge, in one dimension while in the second dimension proteins were separated on the basis of molecular weight. Autoradiograms were obtained at -70°C.

Altogether 10 experiments have been performed, six at 12°-13°C and four at 15°C. In each experiment four eyes, incubated in \(^{35}S\)-methionine at the projected time of light onset (CT 0), were compared with eyes incubated at projected light offset (CT 12). In a 2-hour incorporation experiment, more than 150 protein spots could be resolved, indicating that the intact eye is metabolically quite active in synthesizing new proteins. In four out of six experiments at 12°-13°C, a relatively intense protein spot (pI = 7, m.w. = 25,000 daltons) is present at CT 12 and
Further analysis of results is in progress.

References:

City of Hope, Duarte, California.

165. CIRCADIAN ACTIVITY IN THE ISOLATED ABDOMINAL GANGLION
Investigators: Susan L. Mailheau, Floyd R. Schlechte, Felix Strumwasser

Last year we described a neuronal spike sorting system that could separate and count distinct waveforms in a multiunit record over time (Biology 1982, No. 169). With this system we are studying the long-term pattern of nerve impulses generated simultaneously by individual axons within a nerve trunk of an isolated ganglion.

This report is concerned with long-term recordings from the pericardial nerve in the isolated abdominal ganglion. Preparations are organ cultured in a modified Eagle's minimum essential medium under conditions of constant darkness and controlled temperature (~14°C). The activity of the pericardial nerve is recorded with a monopolar tunnel electrode and the incoming waveforms are digitized, classified and separated into five distinct units using an on-line Apple II Plus computer system. The unit counts are analyzed for periodicity with multiple power spectral analysis.

The data reported here have been obtained from the seven most carefully controlled organ cultured ganglia. The duration of time that these preparations were studied ranged from nine to 32 days. The histogram of periods obtained from 27 power spectral analyses shows a normal distribution of single unit cases with a mean period of 26.0 (±11.0) hours. 56% of these units had periods in the range 18 to 29 hours with a mean period of 23.4 (±3.7) hours. We therefore conclude that the isolated abdominal ganglion contains neuronal circadian oscillators that are expressed in the absence of external zeitgebers.

166. MAPPING NEURONAL METABOLIC ACTIVITY IN THE EYE OF APLYSIA WITH HIGH RESOLUTION [3H]2-DEOXY-GLUCOSE AUTORADIOGRAPHY
Investigators: Karen Herman, Felix Strumwasser

We have been looking for circadian or light-induced variations in the activity of neurons in the Aplysia eye using the 2-deoxyglucose (2-DG) method for mapping neuronal activity. Most of our recent efforts have gone into determining which modifications of the 2-DG technique permit the highest resolution autoradiography and the best visualization of the cells. We have found that to block diffusion of 2-DG and its soluble metabolite, completely dry autoradiographic techniques must be used. Thus, published modifications of the 2-DG method using liquid nuclear track emulsion (Sejnowski et al., 1980) do not retain all the soluble label in place. Best resolution was obtained with freeze-substituted, epoxy-embedded eyes that were sectioned and exposed to dry nuclear track emulsion according to Buchner and Buchner (1983). In those specimens, the photoreceptor microvilli and nuclear layers were heavily labeled, whereas silver grains were less frequent over the pigmented layer. In the pigmented layer, radially oriented "spokes" of heavily and lightly labeled cells are visible. The sharp demarcations between the heavily and sparsely labeled areas suggest that good resolution, perhaps less than 3 µm, has been obtained.

Nonetheless, the method has its drawbacks, since the dry sections cannot be stained or realigned with the autoradiogram. Thus, we have tried a modification of the liquid emulsion technique so as to visualize both the cells and autoradiogram. Epoxy sections of the freeze-substituted eyes were soaked in water for 3 hr at 50°C so as to remove label which could diffuse out and obscure detail when the sections are coated with liquid emulsion. Autoradiograms of these sections showed considerable loss of label when compared to the dry autoradiograms, but the pattern of labeling was very similar. Since many silver grains were present over areas lacking glycogen, such as the microvilli, these autoradiograms appear to reflect the distribution of soluble label trapped in the plastic, as well as insoluble label incorporated
into glycogen. Resolution in these autoradiograms is better than in liquid emulsion autoradiograms of sections that were not pre-soaked, but not as good as in autoradiograms produced under completely dry conditions. Nonetheless, these autoradiograms indicate that the lightly labeled "spokes" in the pigmented layer are the pigment support cells, whereas heavier concentrations of grains overlie photoreceptor and ciliary cells. Thus, a combination of dry and liquid emulsion techniques has been useful in resolving 2-DG labeling patterns over small cells. We have used these techniques to produce autoradiograms of eyes at the peak and trough of compound action potential activity and under light or darkness. Analysis of these autoradiograms is under way.

References:

167. REGIONAL SPECIALIZATION OF NEUROSECRETORY AND PHOTORECEPTOR CELLS IN THE EYE OF APLYsIA

Investigators: Karen Herman, Felix Strumwasser

Except for the cephalopods, most molluscan eyes are small and superficially appear rather primitive. The eyes of Aplysia fit this description, but on closer examination we have found that they have numerous regional specializations, some of which are similar to those found in larger, more well-developed eyes. The Aplysia eye appears to be divided into dorsal and ventral regions with a horizontal line through the optic nerve head forming a boundary between the regions. One cell type, the neurosecretory cell, is completely absent from the dorsal region. Neurosecretory cells are most abundant in a horizontal band located just ventral to the optic nerve head, but are also present in the ventral retina below this band. Coincident with the band of neurosecretory cells, there is a horizontal band of extensive and complex neuropil. The neuropil is also present, but less conspicuous, throughout the ventral retina, but is nearly absent from the dorsal retina. The dorsal retina appears to be a "simplified" retina consisting only of photoreceptors, ciliary cells, and pigment support cells. In the dorsal retina, photoreceptor axons can be observed coursing towards the optic nerve head, but it is not known whether they exit there or make synaptic contacts with cells in the vicinity of the optic nerve head.

Photoreceptors in the dorsal and ventral retina differ significantly in their morphology. The photoreceptor microvilli are much longer in the dorsal retina, and their cell bodies, which are filled with photic vesicles, are also much longer. Tangential sections through the pigmented portions of the photoreceptors show that in the ventral retina, the photoreceptor cross sections are roughly oval, whereas in the dorsal retina, they are highly elongate, U-shaped or star-shaped. The cross-sectional area of the dorsal photoreceptors is as much as four times greater than that of the ventral photoreceptors.

Dorsal and ventral pigment support cells also differ in their morphology. The pigment support cells surround the photoreceptors so that adjacent photoreceptors are not in direct contact. In the ventral retina, only one pigment support cell separates adjacent photoreceptors, whereas dorsally, the photoreceptors are separated by a double layer of pigment support cells. The pigment layer is also radially longer in the dorsal retina.

What functions might these regional specializations have? The dorso-ventral differences in photoreceptor morphology may be explained in terms of the relative brightness of the upper and lower visual fields. Dorsal photoreceptors, which view the relatively dimly lit lower visual field, have modifications which would be expected to increase their sensitivity, but limit their resolving power. In contrast, the morphology of the ventral photoreceptors suggests lower sensitivity, but greater acuity. The asymmetric distribution of the neurosecretory cells is less easily explained. An intriguing possibility is that these cells are located ventrally for the purpose of tracking the image of the sun, which in combination with the eye's circadian oscillator, could constitute a navigational system.
168. BINDING OF CERTAIN LECTINS TO APLYSIA PHOTORECEPTOR CELLS

Investigators: Karen Herman, Felix Strumwasser

We have used lectins, which bind to specific saccharide residues of glycoproteins or glycolipids, to characterize plasma membranes and intracellular glycoconjugates of Aplysia photoreceptors. In the first set of experiments, the binding of lectins to cultured photoreceptors with intact plasma membranes was examined. Fixed cells were incubated with eight different fluorescein-conjugated lectins (WGA, MPA, Con A, DBA, SBA, PNA, BSA I, BSA II). Both WGA and MPA bound to the photoreceptor microvilli, but the other lectins did not. Fixed, cultured photoreceptors were also incubated in WGA-ferritin, which bound to both the microvilli and plasma membrane. A similar assessment of the distribution of binding has not been made with MPA. The specificity of lectin binding was demonstrated in control experiments, in which addition of the appropriate sugar blocked binding. Binding of WGA suggests that the photoreceptor microvilli and plasma membrane contain glycoconjugates bearing N-acetyl glucosamine or sialic acid residues (Greenaway and Levine, 1973). Similarly, the binding of MPA suggests that galactose residues are present (Bausch and Poretz, 1977), but the failure of BSA I to bind suggests that they are not in the terminal position of the sugar chain (Hayes and Goldstein, 1974).

In another set of experiments, the cell membranes were permeabilized according to Chiu and Strumwasser (1981), so that lectins could bind to intracellular glycoconjugates. Even after permeabilization, MPA-fluorescein was observed only on the photoreceptor microvilli, but WGA bound strongly to a band of cytoplasm between the absorptive pigment and nucleus of the photoreceptor, as well as the microvilli. The rest of the photoreceptor soma was only lightly-stained by fluorescein-conjugated WGA. The only other lectin that bound to photoreceptors following permeabilization was Con A. Con A bound to the cell bodies of the photoreceptors, but not the microvilli. Binding of WGA and MPA was blocked, and Con A binding strongly reduced, by adding the appropriate sugars.

The patterns of lectin binding to the photoreceptors provide some evidence as to the organelles from which the microvilli and plasma membrane might originate, or be degraded in. The strong binding of WGA between the nucleus and absorptive pigment suggests that the microvilli may arise from organelles in this region, which appears in electron micrographs to be rich in rough endoplasmic reticulum. However, in preliminary electron microscopic experiments, WGA-binding was observed only in multivesicular bodies in this region. Nonetheless, the failure of WGA and MPA to bind to most of the photoreceptor soma, which is dominated by photic vesicles, suggests either that the rhabdomal microvilli are not derived from these vesicles (Eakin and Brandenburger, 1975), or that glycosylation of the microvilli occurs via cell surface glycosyltransferases.

References:


169. EFFECTS OF MUTAGENS/CARCINOGENS ON THE CIRCADIAN RHYTHM OF APLYSIA EYE

Investigators: John C. Woolum, Felix Strumwasser

We have previously shown that X-rays can inhibit the circadian modulation of the compound action potentials (CAPs) from the eye (Woolum and Strumwasser, 1980). Because the dose of X-rays required is rather small (about 6 krad), the structures that are being affected by the X-rays must be rather large and present in small numbers. One possibility is that nucleic acid molecules are important in generating the circadian rhythm. We have studied the effects of a number of mutagens/carcinogens which may have an effect on the cell's nucleic acids. Ethidium bromide which is known to bind to nuclear DNA and to uncoil and have effects on transcription of mitochondrial DNA (within minutes) lengthens the circadian period by several hours if it is administered at 30 µm continuously. 5-Fluorouracil (but not 5-fluoro-deoxyuracil) at
5 mM has a similar period-lengthening effect. A 5-min treatment of an eye in N-methyl-N’-nitro N-nitroso guanidine (100 µg/ml), a potent methylating agent and mutagen, inhibits the circadian modulation of the CAPs without affecting other membrane functions such as size and number of spontaneous CAPs or production of CAPs by light. Its action is quite similar to the action of about 40 krad of X-rays. Other mutagens/carcinogens such as 4-nitroquinoline n-oxide (1-hr treatment at 100 µg/ml), acriflavine (continuous treatment at 30 µm), and N-nitroso methyl urea (continuous treatment at 300 µg/ml) all inhibit the circadian modulation of the CAPs without inhibiting production of CAPs. These agents may affect the circadian oscillator through their action on nucleic acids but other effects such as on membrane proteins or other enzymes cannot be excluded without further experiments.

Reference:

170. MEMBRANE-ACTIVE AGENTS THAT AFFECT THE CIRCADIAN RHYTHM IN THE EYE OF APLYSIA

Investigators: John C. Woolum, Felix Strumwasser

We have previously observed that agents which decrease Ca\(^{2+}\) uptake by internal membranes lengthen the circadian period of CAPs from the eyes. It has been shown previously that a carrier of K\(^+\) ions in membranes (valinomycin) can cause phase shifts in circadian rhythms in other systems. We have now studied several other agents that affect K\(^+\) transport through membranes as well as other agents known to have effects on membrane-bound proteins. Tetraethylammonium, 4-amino pyridine and quinine are known to block transport of K\(^+\) ions through K\(^+\)-specific channels. All three of these agents inhibit the circadian modulation of CAPs from eyes at concentrations equal to or somewhat less than those used to block K\(^+\) channels. The circadian rhythm returns phase delayed when these agents are washed out. Another agent, vanadate, which is known to inhibit activity of membrane-bound enzymes (as well as other enzymes) when administered continuously to eyes at 100 µM lengthens the circadian period of CAPs. Evidence from these experiments and previous experiments points to the importance of membranes in the expression and/or modulation of the circadian rhythm.

PUBLICATIONS

171. THE ORGANIZATION OF CORTICAL VISUAL AREAS IN A STREPSIRHINE PRIMATE, GALAGO SENEGALENSIS

Investigators: John M. Allman, Evelynn McGuinness

The order Primates is divided into two suborders: haplorhines (tarsiers, monkeys, apes, and man) and strepsirhines (galagos, lorises, and lemurs). In order to trace the evolutionary history of the cortical visual areas, we have sought to determine which areas are present in both suborders. We have mapped the representations of the visual field with microelectrode recordings in the visual cortex of a strepsirhine primate, Galago senegalensis. Our results indicate that the primary visual area (V-I), the second area (V-II), and the middle temporal (MT), the dorsolateral (DL), the medial (M), the ventral posterior (VP), the ventral anterior (VA) visual areas are all present in Galago. Previous data indicate that these areas are present in New World monkeys. There is also substantial evidence from other laboratories that most, if not all, of these areas also are present in Old World monkeys. Galago also possesses extensive, visually responsive inferotemporal and posterior parietal cortical regions, but, as in monkeys, receptive fields of neurons in these regions do not appear to be organized in obvious visuotopic maps.

Recently Horton and Hubel (1981) have reported that the system of cytochrome oxidase stained patches in layers II and III of striate cortex are present in Galago as well as New and Old World monkeys but are not present in nonprimates such as cats and tree shrews. The results suggest that there exists a distinctive primate pattern in the functional organization of striate cortex.

Taken together these similarities in the organization of striate and extrastriate visual cortex in strepsirhine and haplorhine primates suggest that these features were present in the common ancestor of these living forms, which would have been a primitive primate living during the Eocene period 36 to 55 million years ago. These early primates possessed large frontally directed eyes and an expanded posterior neocortex; they were small, probably nocturnal, visually predatory, insectivorous creatures that lived in the fine terminal branches of trees (Cartmill, 1974; Radinsky, 1967; Simons, 1972). The comparative neurobiological data suggest that many of the distinctive features of the primate visual system developed in these early primates.

References:

172. ANTAGONISTIC DIRECTION AND VELOCITY SPECIFIC SURROUND MECHANISMS IN AREA MT IN THE OWL MONKEY

Investigators: Francis M. Miezin, Evelynn McGuinness, John M. Allman

It is well known that most area MT neurons are strongly selective for the direction of stimulus motion. We wished to know how MT neurons behaved when stimuli were presented against moving textured backgrounds such as occur commonly in natural stimulus conditions. We have explored the receptive field structure and directionally selective mechanisms in MT using a newly developed microprocessor-based video display that can present simultaneously bars and random dot patterns moving in different directions. In nearly all MT neurons tested, when a bar moving in the cell's preferred direction is presented against a background of random dots moving in the same direction, the response is greatly reduced relative to the response to the bar moving on a background of stationary random dots. When the background is moved in the opposite direction, the response to bar stimulation in the preferred direction is...
either less inhibited or in some cases strongly facilitated.

In a related set of experiments we sought to map the extent and nature of antagonistic mechanisms in the surround. By continuously driving MT neurons by stimulating the conventionally defined receptive field and by simultaneously moving fields of random dots in the surrounding visual field, we have found that the antagonistic surrounds are typically at least eight times the diameter of conventionally defined receptive fields. Thus the antagonistic surround for the average receptive field in MT extends over more than a hemifield. These surround effects are often directionally selective with the preferred direction of the center being the direction of maximum inhibition in the surround. The surrounds are also velocity sensitive with maximum inhibition occurring when the surround is stimulated at velocities close to the preferred velocity for the center. Nakayama and Loomis (1974) predicted the existence of velocity sensitive surrounds and suggested that such a mechanism would serve to detect discontinuities in optical flow revealing surfaces at varying depths from the observer. These antagonistic velocity and direction-selective mechanisms provide an ongoing comparison between movements occurring locally in the visual field and more global retinal image movements such as would occur during eye, head or body movements. Thus they may be related to the mechanisms that accomplish the perceptual stability of the visual world as well as to the enhancement of figure-ground discrimination.

Reference:

173. ONTOGENY OF MONAMINERGIC RECEPTORS

Investigators: Gosta Jonsson*, Takuji Kasamatsu

A preliminary result of the present study appeared in last year's Biology Annual Report (Biology 1982, No. 173). Endogenous catecholamines (norepinephrine [NE] and dopamine [DA]) in the visual cortex gradually increased with postnatal age, showing about 12-13-fold increase in their content from the newborn to the adult. $^3$H-di-hydroalprenolol binding showed a rapid increase from the relatively low value (25% of the adult value) at birth, peaking (150% of the adult value) at the age of 7-9 weeks. The $\beta$-adrenoreceptor binding then stayed relatively constant at the adult value from the age of 11 weeks onward. On the other hand, though endogenous 5-hydroxytryptamine (5-HT) levels at birth were about 20% of the adult value, they thereafter rapidly increased to reach the maximum at the age of 3-5 weeks. The 5-HT level drops down to 50-60% of the adult value between the age of 7 and 13 weeks postnatally. The $^3$H-5-HT receptor binding showed a steep peak at an age of 4 weeks with a value threefold higher than the adult. Thereafter, the 5-HT binding gradually leveled off toward adulthood. The postnatal maturation of monoamine receptors appears to precede that of their nerve terminals in the cerebral cortex.

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174. RESTORATION OF NEURONAL PLASTICITY IN CAT VISUAL CORTEX BY STIMULATION OF THE LOCUS COERULEUS

Investigators: Takuji Kasamatsu, Kazushige Matabe*, Erling Schøller**, Paul Heggelund**

The overwhelming majority of norepinephrine (NE)-containing nerve terminals in the cerebral cortex originate from the NE-containing cells in the brain stem structure, the nucleus locus coeruleus. Last year (Biology 1982, No. 187) we reported that electrical stimulation of NE cells in the locus coeruleus resulted in the restoration of neuronal plasticity in the visual cortex of adult cats and old kittens that had outgrown the postnatal critical period.

We recently made further progress by obtaining the following three findings. First, no effects of stimulation of the locus coeruleus were noted if the visual cortex had been priorly perfused with 6-OHDA, suggesting that the restoration of plasticity after locus coeruleus stimulation is mediated by NE-containing nerve terminals within the visual cortex. Second, the neuronal plasticity thus restored to the matured cortex seemed to continue for at least 3 weeks after stopping stimulation of the locus coeruleus. Third, there was no obvious relationship between the extent of plasticity restored by locus coeruleus stimulation and the age of the animals.
In the present study, the higher availability of NE within the cortex was attained through an increased release of endogenous NE by activation of NE cells in the brain stem, leaving the entire cerebral cortex untouched. Thus, the present results further strengthened our NE hypothesis of visual cortical plasticity.

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175. CHANGES IN NOREPINEPHRINE UPTAKE IN KITTEN VISUAL CORTEX PERFUSED WITH 6-HYDROXYDOPAMINE

Investigators: Takuji Kasamatsu, Kunio Nakai

In the kitten visual cortex perfused with 6-hydroxydopamine (6-OHDA), we have studied the reinnervation of such cortex with norepinephrine (NE)-containing nerve terminals at various time intervals after the initial lesion specific to catecholamine (CA) terminals. As described in Abstract No. 176, we have primarily used CA fluorescence histochemistry and a biochemical assay of endogenous CAs to delimit the extent of chemical lesion of CA terminal fields. Although CA fluorescence histochemistry is a powerful method to visualize the quick reduction in size of the cortical area devoid of CA terminal fields, it is not easy to quantify the distribution pattern as well as the intensity of fluorescence and to compare them among different animals. Alternatively, we started to examine the spatial and temporal distribution of the number of NE uptake sites by measuring the proportion of 3H-NE taken up by NE terminals to that left in the incubation medium (Snyder et al., 1968; Jonsson et al., 1969). Thirty fresh cortical sections (300 μm thick), sliced by a tissue chopper at regular intervals from the site of 6-OHDA perfusion, are taken from deeply anesthetized animals at various times after the end of the 6-OHDA perfusion. In the cortex immediately after the end of 6-OHDA perfusion, the tissue/medium uptake ratio was very close to zero in a cortical area up to 5-6 mm from the perfusion site and then increased quickly to attain the maximum at 10 mm. This profile of spatial distribution of NE uptake sites corresponds well to that visualized by CA fluorescence histochemistry. Currently we are studying how this uptake ratio curve, plotted against the distance from the perfusion site on the abscissa, moves to the left as the time interval increases after the end of 6-OHDA perfusion. In the visual cortex of normal cats, the K_m and V_max of NE uptake were at 6.3x10^-8 ~ 1.7x10^-7 M and 23-42 pg/mg tissue, respectively.

References:

176. REGROWTH OF CENTRAL CATECHOLAMINERGIC FIBERS IN CAT VISUAL CORTEX FOLLOWING LOCALIZED LESIONS WITH 6-HYDROXYDOPAMINE: A FURTHER STUDY

Investigators: Kunio Nakai, Gösta Jonsson*, Takuji Kasamatsu

We have been studying the regrowth of catecholamine (CA)-containing fibers in cat visual cortex. A local cortical area is first perfused with 4 mM 6-hydroxydopamine (6-OHDA) for a week to cause a chemical lesion of CA terminal fields. The animal survives for a variable time period thereafter. Last year we reported results that suggest the rapid regrowth of central CA fibers that re-innervate the CA terminal-degenerated area in the cortex (Biology 1982, No. 178). In order to elucidate changes, regeneration followed by degeneration, in the CA terminal fields within the cortex, we used various methods which include CA histofluorescence, high pressure liquid chromatography for endogenous CAs, light microscopic autoradiography for β adrenoreceptors and electron microscopy of CA terminal boutons. Since the number of β-adrenoreceptor binding sites, as visualized by 3H-dihydroalprenolol autoradiography, increased by about 50% in the cortical area near the site of 6-OHDA perfusion, we are convinced that we are primarily dealing with regeneration of denervated CA terminal fields rather than a simple refilling of the once-emptied CA storage in CA terminals. This view has been further supported by our recent results of an NE uptake study in the 6-OHDA-perfused visual cortex (see Abstract No. 175).

In the present study, 3.3 mM substance P (SP),
an undecapeptide rich in the CNS, injected into the locus coeruleus (LC) region during the survival period, significantly accelerated the rate of reinnervation of the visual cortex by CA fibers. No accelerated reinnervation was seen if SP was perfused directly into the cortical area devoid of CA terminal fields. This facilitatory effect of SP on CA fiber reinnervation does not seem to be mediated through mere excitation of NE-containing cells in the LC, since a muscarinic cholinergic agonist, bethanechol, which is as potent as SP in exciting NE cells in the LC, did not reproduce the accelerated reinnervation by CA terminal fields of the visual cortex devoid of them. Conversely, injection of morphine, which suppresses activity of NE cells in the LC, into the LC region did not affect the reinnervation process. We propose a hypothesis that SP in the CNS may work as part of a built-in repair mechanism for injured NE fibers.

177. MORPHOLOGY OF CATECHOLAMINERGIC TERMINALS IN CAT VISUAL CORTEX

Investigators: Kunio Nakai, Takuji Kasamatsu

This is a continuation of the project which was started two years ago (see Biology 1982, No. 179). Electron microscopy of ultrathin, serial sections obtained from the 6-OHDA-treated visual cortex of adult cats, fixed with glutaraldehyde and osmium tetroxide, revealed that only 29% of identified CA terminal boutons (N=187) make the usual form of synaptic contact with neighboring neuronal elements. The favorite targets were either dendritic shafts or spines. This low proportion of synapse-forming CA terminal boutons was in contrast with the much higher proportion (58%) of synapse formation among nonaminergic terminal boutons in the neighboring area of the same sections. We also studied the CA terminals in the visual cortex of 10-day and 7-week-old kittens. Forty-four percent and 32% of 6-OHDA-labeled CA terminal boutons (N=50, and 50) were found to have the usual form of synapse, respectively. These results are essentially consistent with the previous findings from our laboratory: in the kitten visual cortex fixed with KMnO₄, following glyoxylic acid perfusion, we found that only 20% of thus identified CA terminal boutons made synapses with surrounding neural elements (Itakura et al., 1981). The single largest group (38.5%) of the synapse-forming CA terminal boutons was found in layers II and III, in which not only CA terminals in fluorescence histochemistry but also β adrenoreceptors in β-antagonist autoradiography showed the highest density of distribution among the six cortical layers. Furthermore, most synapses formed by CA terminals in layers II and III were the symmetrical type which is generally thought to be inhibitory (Ribak, 1978; Peters and Proskauer, 1980). Catecholamine released from CA terminals seems to have a dual action in the neocortex: it works as a neurohormone and also as a neurotransmitter through the beginning synapses.

References:
deeper part which was characterized by numerous parallel 5-HT-positive fibers with strong intensity of staining.

Next we examined the effects of 5,7-dihydroxytryptamine (5,7-DHT), a 5-HT related neurotoxin, on 5-HT-positive terminals in kitten visual cortex. Immediately after the continuous perfusion of the cortex with 4 \( \mu \)M 5,7-DHT, which lasted for a week, we found a discrete cortical area devoid of 5-HT terminals near the site of perfusion. The radius of this chemical lesion of 5-HT terminal fields was about 5 mm from the perfusion site and comparable to that seen in the cortex subjected to equimolar 6-hydroxydopamine (6-OHDA) and examined with catecholamine (CA) histofluorescence (Kasamatsu et al., 1981). Using both 5-HT immunocytochemistry and CA fluorescence histochemistry, we are going to examine the possible effects of 6-OHDA on 5-HT terminal fields and those of 5,7-DHT on CA terminal fibers in cat visual cortex.

References:

179. CNS ELECTROPHYSIOLOGY IN A VISUALLY PREDATORY ARACHNID

Investigators: David W. Sivertsen, Cecile B. Huling*

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 (Forster, 1982).

Previous work has quantified behavior (Land, 1972), receptor properties, and anatomy (Hill, 1975). No one has previously recorded from the central nervous system. Visually predatory spiders have the advantage of simplicity, characteristic of invertebrate preparations, yet are capable of complex visual behaviors. Phidippus johnsonii, a salticid found in the campus gardens, is well-suited for study.

Salticids have eight eyes. One pair is largely vestigial, two pair are primarily concerned with orienting toward the target, and the anterior medial pair (AM) performs complex analysis. The AM eyes have a 2° wide by 20° high retina. The receptors are ordered into four distinct layers. This array takes advantage of the focal length versus wavelength aberration to extract color information from green to ultraviolet (Yamashito, 1976). The layers are also tiered along the 2° width.

Although the lenses of the principal eyes are fixed, 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. 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.

We have found two novel properties with single and multiunit recording in the CNS. The first is receptive fields which are "space-mapped." These cells respond to stimuli in a constant spatial region that subtends an area much larger than the retina.

The second novel property is monocular depth perception. Many visual receptive fields in spiders and vertebrates subtend a constant angle, independent of stimulus distance. In P. johnsonii, we have found "size-constant" cells. The angular subtense of the receptive field diminishes with distance, with the physical size remaining relatively constant. This processing requires depth information. The probable mechanism is the tiering of the retinal layers. Translational eye movements result in receptors being present at different distances along the optical axis. For near-field vision, objects at different distances have differing points of best focus.

Both of these phenomena require eye movements. Current work includes construction of a retinal monitoring system to verify these hypotheses.

References:

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PUBLICATIONS


Summary: "From behavior to neuron" is the main theme of research done in this laboratory. The study of sound localization in the barn owl shows that this theme is feasible even in vertebrates. During the past year we have made much progress in neurophysiological and anatomical tracing of the neural pathways involved in sound localization. The owl uses two different acoustic cues, binaural intensity and phase differences, to localize sound in, respectively, vertical and horizontal planes. These two cues seem to be processed in two separate brain stem auditory pathways. This separation starts right at the beginning of the pathways, the cochlear nucleus, which consists of two anatomically distinct subdivisions. Neurons in one division encode only the phase of tonal stimuli and those in the other division encode only the...
intensity. Injection of local anesthetic into one division affects processing of either phase or intensity information but not both. The separation seems to involve differences in synaptic mechanisms between the two divisions. Each primary auditory fiber innervates both divisions via two separate collaterals. Histological studies clearly show that the two divisions have different types of synapses. Thus, we have reached the point where biophysical and cell biological studies can be directly linked to behavioral observations.

180. USING LOCAL ANESTHETICS TO FUNCTIONALLY DISSECT THE OWL'S AUDITORY SYSTEM

Investigators: Andrew Moiseff, Terry T. Takahashi, Masakazu Konishi

Owls accurately determine the elevation and azimuth of sounds by processing interaural intensity differences and interaural time differences, respectively. Physiological and anatomical studies suggested that interaural time and interaural intensity differences were processed along separate neural pathways. Nucleus magnocellularis and its target, n. laminaris, made up the first levels of a "time processing pathway"; nucleus angularis and one of its projection areas, nucleus ventrale lemniscus lateralis (pars posterior), made up the first levels of an "intensity processing pathway." To confirm the independence of these two pathways, we decided to inactivate one of them while leaving the other one intact. We temporarily "turned off" selective brain stem auditory nuclei by applying lidocaine, a local anesthetic, through a microelectrode. The lidocaine blocked neural activity in the vicinity of the microelectrode for 5-50 minutes. A second microelectrode was placed in the external nucleus of the inferior colliculus, the region of the midbrain containing "space selective units."

Normally, space selective neurons are highly selective to narrow ranges of interaural time differences and interaural intensity differences. When lidocaine is injected into nucleus magnocellularis or nucleus laminaris, however, the space selective neurons remained selective to interaural intensity differences but lose their high selectivity to interaural time differences. When the effect of the local anesthetic wore off, the space selective units regained their original sensitivity to time differences.

This confirmed that nucleus magnocellularis and nucleus laminaris were part of the time processing pathway, and, since the anesthetic affected time selectivity but not intensity difference selectivity, it also supported the notion that time and intensity differences are processed along separate anatomical pathways. We are now anesthetizing nuclei in the presumed intensity processing pathway to further demonstrate the validity of our hypothesis.

181. HIGH FREQUENCY PHASE CODING IN THE COCHLEAR NUCLEUS OF THE BARN OWL

Investigators: W. Edward Sullivan, Masakazu Konishi

Ongoing time of phase differences between the two ears is utilized by barn owls to localize high frequency sounds (5-9 kHz) along the horizontal axis. For the auditory system to measure time differences in high frequency waveforms, waveform timing must be preserved at the monaural level. Since studies of mammalian auditory systems have failed to demonstrate phase or time coding at frequencies greater than 4 to 5 kHz, we investigated phase coding in the owl's cochlear nucleus to see if waveform timing is preserved at frequencies where binaural time sensitivity can be shown.

Single unit responses were obtained from output fibers of the nucleus magnocellularis. This nucleus is known to project bilaterally to n. laminaris, the first brain stem site where binaural time sensitivity can be found. Best frequencies (BFs) of units ranged from 2.0 to 9.0 kHz. Virtually all units with BFs from 2 to 6 or 7 kHz showed clear phase coding. In contrast to cat and monkey data, there does not seem to be a dramatic decline in phase locking ability over this range. Further, clear examples of phase coding could be seen in the range of 7 to 9 kHz. At these frequencies the period length is from 110 to 140 microseconds. Analysis of cycle histogram peak width shows that these neurons reliably respond within a range of 25 to 40 microseconds about the mean response phase.
These observations indicate that the owl's auditory system is capable of encoding waveform timing at frequencies over an octave higher than the maximum frequencies where phase locking is seen in mammalian acoustic nerve fibers. This observation provides a basis for the earlier finding that owls can use binaural time or phase information for localization of high frequency sounds. It also raises the question of how the auditory system is able to code stimulus timing with an accuracy in the range of tens of microseconds.

102. FLUORESCENT LATEX MICROSPHERES: A NEW RETROGRADE TRACER FOR NEUROANATOMY

Investigators: Lawrence C. Katz, Andreas Burkhalter, William J. Dreyer

The use of retrograde axonal transport has provided an increasingly detailed picture of central nervous system connectivity. We have developed a new class of retrograde tracer: intensely fluorescent, minute latex microspheres, or "beads" (0.02-0.2 µm diameter). These beads have several unique properties that distinguish them from other tracers currently in use. Injections into the brain produce small, highly circumscribed injection sites (75-100 µm diameter) with no visible tissue lesion. Beads are neither degraded nor do they diffuse out of labeled neuronal somata, even after long survival times. Unlike any fluorescent tracer currently used, these beads are visible in the electron microscope, and their fluorescence is essentially non-fading even after prolonged illumination or storage.

When beads are injected into the rat primary visual cortex (area 17), labeled cell bodies are seen within area 17, in other cortical areas, and within the thalamic input nuclei to visual cortex. Beads are taken up and transported by broken fibers: injections into the corpus callosum labeled large numbers of cells within cortex.

The highly restricted injection sites produced by this tracer make it particularly useful for studies of local circuitry within and between restricted brain areas. In addition, the relative permanence of the label makes beads attractive for developmental studies on the growth and reshaping of axonal connections. Finally, it may be possible to link specific molecules or antibodies to the beads and thereby create highly specific retrogradely transported probes to study the connectivity of specific neuronal subclasses.

103. INTRALAMINAR CONNECTIVITY IN THE RAT VISUAL CORTEX

Investigators: Andreas Burkhalter, Lawrence C. Katz

Within the primary visual cortex (area 17) of mammals, neurons exhibit response properties not evident in lower visual centers, such as selectivity for the orientation, speed, direction and length of visual stimuli. In order to understand how such properties are elaborated, it is necessary to understand the intrinsic circuitry of the visual cortex. We have approached this problem using a new retrograde tracer, fluorescent latex microspheres (see Abstract No. 182) whose properties allow us to study the connectivity of individual cortical layers.

We injected small volumes of tracer into layer IV (the primary input layer) of rat area 17. This produced a stereotyped pattern of retrogradely labeled neurons in layers V and VI. When viewed in coronal section, a small injection site in layer IV (80-100 µm diameter) resulted in a column of labeled cells, about the width of the injection site, which descended through layers V and VI. At the very bottom of layer VI (bordering on the white matter) neurons were very heavily labeled, and labeled cells extended for hundreds of micrometers in both the anterior-posterior and medial lateral directions. These cells occurred in distinct patches or clusters, and their cell bodies often were fusiform in shape. We studied the detailed morphology of these cells by preparing in vitro brain slices of brains that had been previously injected with microspheres. Intracellular injection of the fluorescent dye lucifer yellow into microsphere labeled neurons revealed the dendritic and axonal arborizations of these cells. The dendrites were heavily spined, and their pattern of branching resembled that of a horizontally lying pyramidal cell.

The fact that a small injection in layer IV extensively labels neurons in layer VI implies a striking degree of convergence from layer VI onto...
layer IV. We are currently testing the idea that the layer VI cells may be involved in an inhibitory lattice system that helps to shape the response properties of layer IV cells.

184. MICROCIRCUITRY OF VISUAL CORTEX STUDIED IN IN VITRO BRAIN SLICES

Investigator: Lawrence C. Katz

The mammalian primary visual cortex (area 17) is organized to process incoming thalamic information in a stereotyped manner and to send various outputs to other brain areas. A necessary step in understanding how information is processed is understanding the arrangement of the axons and dendrites of cortical neurons; this delineates the paths by which information in one area of cortex can reach other areas. To this end I have been studying the dendritic and axonal arborizations of intracellularly stained neurons in an in vitro brain slice preparation of cat visual cortex.

The brain slice technique has several advantages over other techniques for the study of cortical circuitry. Conventional stains for cells, such as the Golgi method, often do not reveal the complete axonal arborization of a cell. Intracellular staining in the living animal is very difficult due to brain pulsations. The in vitro approach gives complete visualization of dendrites, axons and axon collaterals contained within a 400 μm thick brain slice. Because the preparation is mechanically very stable, 20-30 neurons can be intracellularly stained in each experiment.

I have begun investigating whether neurons that have specific cortical projections outside area 17 have specific patterns of axonal arborizations within area 17. In these experiments, I have employed fluorescent red latex microspheres (see Abstract No. 182) as a retrograde marker to define particular neuron subclasses based on their efferent connectivity. Microspheres are injected into the corpus callosum of a cat; two days later brain slices are prepared from area 17. Neurons are then intracellularly injected with the fluorescent dye lucifer yellow; those which project across the corpus callosum contain microspheres (red) in addition to lucifer yellow. Since the efferent connectivity of a neuron has some relationship to that neuron's functions, I hope that this approach will lead to a more functionally relevant scheme of cortical neuron classification.

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microelectrodes for recording extracellular neural activity at thalamic and tectal auditory relay stations to investigate the changes in neuron responsiveness to an auditory stimulus that has been made to acquire behavioral significance.

In recent years much attention has been given to the central catecholamine systems as potential candidates for being the anatomical basis of the brain reward processes. The evidence for this view is based on results that show an overlap between the reward sites identified in terms of self-stimulation behavior and the distribution of catecholamine cell bodies, axons, and terminals. In addition, pharmacological results indicate that drugs which increase the amount of norepinephrine or dopamine at the synapse, or which potentiate their action through an increase in their duration in the synapse, facilitate self-stimulation behavior, whereas drugs with the opposite actions on these two catecholamines depress self-stimulation behavior. Other evidence indicates that these two transmitters are released during the brain reward process. Yet, the question of the anatomical pathway responsible for the brain reward is not resolved because lesions of these pathways have proved ineffective in abolishing responding for the brain reward.

We have approached the question of the catecholamine’s role by focusing not exclusively on the depletion of these substances produced in forebrain regions when the animals are given a neurotoxin specific to these transmitters, but on the elevation of norepinephrine that is induced in the brain stem only when the neurotoxin is given shortly after birth. Rats with electrodes implanted in that region self-stimulated at much higher rates than control animals, suggesting that the damage to the catecholamine systems induced by 6-hydroxydopamine had produced a potentiation of the brain reward in the dorsal pons. In these animals, the pontine elevation of norepinephrine levels was correlated with the partial or total destruction of the norepinephrine-containing cell bodies of the dorsal pons A6-A4 and with the presence in that region of an extended and abnormally dense network of catecholamine terminals. Our current studies aim to determine whether the enhanced reward in the dorsal pons of the neonatally 6-hydroxydopamine treated animals was related to the presence of the dense network, or to the destruction of noradrenergic cell bodies in that region removing inhibitory regulation normally present, or to overefficacy of the less injured dopamine pathways.

We are also examining the basis of brain reward at sites associated with the dopamine systems. In these studies, the rats have been treated neonatally with 6-hydroxydopamine and a pretreating agent, desmethylimipramine, to restrict the action of the neurotoxin to the dopamine systems. In adulthood, these animals are implanted with stimulating electrodes directed to the substantia nigra and the ventral tegmentum, two structures which contain the cell bodies of the dopamine systems A9 and A10. The selective depletion of dopamine in the forebrain of these animals did not make any difference in the pattern of self-stimulation in these animals. Nevertheless, the A9 and A10 cell groups were heavily damaged, and from a pharmacological standpoint, the evidence strongly implicates dopamine in the brain reward process at these sites. For these sites, we are examining the possibility that compensatory processes may have been activated after the neonatal lesioning procedures and that these processes may have recruited the noradrenergic innervation present at the dopamine sites to mediate the brain reward. Another possibility is that the brain catecholaminergic systems are not the primary factors in brain reward but instead modulate the primary action on brain reward of other non-catecholaminergic systems.

The primary thrust of the neurophysiological learning studies is directed toward the identification of changes in neuron responsiveness to stimuli that become behaviorally significant. We are using a Pavlovian paradigm with two tones of different frequencies as the conditioned stimulus and the neutral stimulus, respectively, and food as the reward, to analyze the basis of the "associative" changes already identified in the medial geniculate and the deep layers of the superior colliculus. Here we are examining the possibility that in the deep layers of the superior...
colliculus, the changes, though of relatively short latencies, may nevertheless be related to the animal's learned behavior, even if this relationship is in the nature of an anticipatory response preceding the motor output. With respect to the changes in the medial geniculate, we are examining the relationship of these changes to the changes in the superior colliculus. Our goal is to delineate the circuitry at the thalamic and midbrain level wherein "associative" neural changes can be observed and then to trace the connections between them and those presumed to occur in higher integrative structures such as the hippocampus.

185. THE ANATOMICAL AND BIOCHEMICAL BASIS FOR THE POTENTIATION OF BRAIN REWARD IN RATS IN WHICH THE CATECHOLAMINE SYSTEMS HAD BEEN LESIONED NEONATALLY

Investigators: Mamoru Umemoto, S. Kurumiya*; Toru Itakura**, Marianne E. Olds

In animals treated on days 3 and 5 after birth with 6-hydroxydopamine (6-OHDA) injected bilaterally into the lateral ventricles to lesion the catecholamine (CA) systems but also to induce noradrenergic hyperinnervation of the dorsal pons, the brain reward process tested in adulthood with the self-stimulation model was greatly enhanced. Although in different regions of the brain the effect of 6-OHDA on catecholamine content is known, the use of different doses, different routes of drug administration and different schedules make it necessary to assess in each experiment the brain changes induced by the treatment, if their effect on behavior is to be understood. In the biochemical experiments, animals treated identically with the neurotoxin to those that showed "enhanced reward" were used. Samples of brain tissue obtained with a punch procedure were taken from frontal cortex, caudate nucleus, hypothalamus, and dorsal pons. The results show that norepinephrine (NE) and dopamine (DA) were at 26% and 25%, respectively, of control values in the lesioned animals, at 28% in caudate, at 32 and 56%, respectively, in hypothalamus. In the dorsal pons samples, the levels of NE were at 226% of controls and the DA levels were not detectable.

The brains of animals treated neonatally with 6-OHDA in the lateral ventricles, implanted with electrodes in the pons, and shown to be self stimulators, were processed with the glyoxylic histochemical fluorescence method for visualization of CA innervation around the electrode tip. The results showed extensive destruction of CA cell bodies in the dorsal pons. In some test animals no cell bodies were seen, in others, the number was substantially reduced. In this same region the density of CA terminals was increased and covered a larger area than in controls. These findings demonstrate that the activation of the pontine brain reward processes is compatible with massive damage to the adrenergic cell groups A6-A4, but do not resolve the question of whether this is due to the presence of an abnormally dense catecholaminergic network, or to some form of compensation induced by the neonatal injury, or to mediation by non-CA neurons.

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186. EFFECTS OF CHEMICAL LESIONS OF THE Dopamine SYSTEMS ON REWARDING EFFECTS OF BRAIN STIMULATION AT Dopamine ASSOCIATED SITES, AND ON REGIONAL CATECHOLAMINE CONTENT OF THE RAT BRAIN

Investigators: Mamoru Umemoto, Marianne E. Olds

The evidence in the literature suggests that an increase of activity in the dopamine systems originating in the A9 cell group of the substantia nigra and the A10 cell group of the ventral tegmentum may constitute the critical neurophysiological events in the brain reward processes. We have examined this idea in animals that were treated at birth with the neurotoxin 6-OHDA injected in the ventricles after pretreatment with desmethylimipramine to lesion the dopamine systems. The reason for selecting the neonatal intraventricular route was that it permits more radical lesions than is possible when the neurotoxin is given in adulthood. These subjects became hyperreactive to novel stimuli, but the lesions had no effects on the frequencies with which brain reward was obtained in the areas where the dopamine cell bodies lay or in the regions of their terminals. Biochemical assays of dopamine and norepinephrine levels in frontal cortex and caudate nucleus of the lesioned animals showed a 90% reduction of dopamine content, whereas
the norepinephrine content was at control levels. Apparently the dopamine containing terminals of these regions were not essential for the brain reward there or in the areas of origin of these two dopamine systems, the substantia nigra and the ventral tegmentum. Conceivably, these dopamine systems are crucial for the rewarding properties to be produced, but owing to the lesioning having been done early in life, compensatory mechanisms were activated that rendered related systems—perhaps the noradrenergic innervation in cortex, ventral tegmentum, and substantia nigra—capable of mediating the rewarding effect under these unusual circumstances.


Investigators: Mamoru Uemoto, Marianne E. Olds

Although the biochemical effects of lesioning the dopamine systems in adulthood or neonatally are well established, the morphological changes induced are less clear. In view of results obtained in this laboratory which showed that the neonatal lesioning of the dopamine systems induced hyperreactivity in the adult rat but had no effects on the distribution or nature of reward at sites associated with the dopamine system, it seemed important to examine the morphological changes induced by the neonatal treatment in the regions surrounding the electrodes that yield brain reward. Experimental animals were obtained by injecting 6-OHDA into the lateral ventricles on days 3 and 5 after birth, after pretreatment with desmethyl-imipramine to restrict the action of the neurotoxin to the dopamine systems. Control animals were injected with saline in the ventricles after desmethyli-mipramine. After testing for hyperreactivity and brain reward, some animals from each group were selected for the histochemical fluorescence studies. The method used was the glyoxylic technique for visualizing catecholamine-containing cell bodies, axons, and terminals. The results show that in the experimental animals the dopamine-containing cell bodies of the A9 group were destroyed by the neonatal lesioning and the same type of cell bodies in the A10 group were markedly reduced in number. However, in the region surrounding the electrode tip, CA-containing varicosities could still be seen at high magnification, which may represent the noradrenergic innervation of the substantia nigra and the ventral tegmentum, thus verifying the biochemical finding that in the experimental animals the levels of norepinephrine were not affected by the neonatal treatment. In the medial frontal cortex, the deep layers were devoid of catecholamine varicosities and this was where the electrode was implanted, whereas the shallow layers contained such terminals. These results verify the finding that the neonatal lesioning of the dopamine systems can be specific to those systems and is capable of inflicting severe damage to the cell bodies, with the A9 group apparently more sensitive to the neurotoxic action than the A10 cell group. This treatment did not produce a catecholaminergic hyperinnervation of the ventral midbrain.

188. COMPARISONS OF THE ASSOCIATIVE NEURAL CHANGES IN THE MEDIAL GENICULATE AND THE SUPERIOR COLLICULUS OF THE RAT TO CONDITIONING PROCEDURES USING FOOD AND REWARDING BRAIN STIMULATION

Investigators: Marianne E. Olds, Toshitasugu Hirano, Jeffrey D. Carpenter

The objective of this experiment is to find out whether some aspect of the associative neural changes observed in these structures expressed some component of the "learned" behavior. Using a differential conditioning paradigm with food as the reward and two tones of different frequencies as the conditioning stimulus (CS+) and the neutral stimulus (CS-), animals implanted with electrodes for recording single and multiple unit activity were trained to move toward a food magazine that dispensed a food pellet 1 sec after the onset of the CS+ when the tone and the food reward were paired. The trained animal did not go to the food magazine when the CS+ and the food were not paired (extinction or habituation) or when the CS- was presented. As the animal learned this task, the amplitude of the auditory responses in the medial geniculate and the superior colliculus (deep layers) increased to the CS+ but not to the CS-.
Although the latencies of the movements were longer than the latencies of the neural changes, it appeared reasonable to assume that, at least in the superior colliculus, the two types of latencies were related in view of the evidence linking the superior colliculus with orientation. One way of examining whether such a relationship was present was to record unit activity with similar conditioning procedures but under conditions where the animal did not move. This is possible when the reward is applied directly in the brain. Hence, after the animal was conditioned with food as the reward, brain stimulation was substituted first in a series of sessions where the animal had to touch sensors on the food magazine with movements very similar to those made to obtain the food reward. This provides the basis for comparing the role of the food and brain stimulus in determining the type of associative neural change produced. Next, the conditioning sessions continued but now the brain stimulus, still paired with the CS+, applied to the animal immobilized with a muscle relaxant, the intention being to examine the contribution of the behavior to the occurrence of associative neural changes. The data are presently being collected.

Professor: Roger W. Sperry
Senior Research Associate: Charles R. Hamilton
Visiting Associates: Evelyn L. Teng, Eran Zaidel
Research Fellow: Polly J. Henninger
Graduate Students: Alice M. Cronin-Golomb, Jay J. Myers
Staff Associate: Betty A. Vermeire
Research Staff: Amy M. Canada, Erika M. Erdmann, Eef Goedemans, Lois E. MacBird,

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Summary: The research continues to be concerned with hemispheric specialization and left-right cross integration in human subjects who have undergone surgical disconnection of the cerebral hemispheres, with special emphasis on subcortical transmission of cognitive information. Correlated investigations, carried out in split-brain monkeys under Dr. Charles Hamilton, carry further the analysis of basic hemispheric interrelations with combined surgical and testing techniques not applicable in human subjects.

189. HEMISPHERIC DIFFERENCES IN THE DISCRIMINATION OF JULESZ MICROPATTERNS

Investigator: Alice M. Cronin-Golomb

Three texture pairs of the type developed by Julesz, i.e., micropatterns sharing the same power spectra or dipole characteristics, were presented tachistoscopically to commissurotomy subjects LB, NG and AA. The texture pairs chosen included two that Julesz deemed "easily discriminable" at less than 200 msec exposure duration, and one "non-discriminable" at this duration. Each texture pair

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was exposed for 200 msec to the right or left visual field for a total of 48 trials, 24 to each field. Subjects were to demonstrate discrimination of the quadrant of the stimulus card containing the micropattern that was different from the micropattern found in the other three quadrants, by pointing to the corresponding square on the grid placed before them.

Subject NG demonstrated a large hemispheric effect: the left hemisphere strongly outperformed the right for one of the "discriminable" pattern pairs, that of dot-pair (dipole) orientation. The right hemisphere's performance was at chance level. LB showed an above-chance discrimination ability for the other "discriminable" texture pair (representing "quasi-collinearity" of dots), but no hemisphere difference. No subject performed above chance level on the "non-discriminable" task (distinguishing "R" from its mirror image), and AA's performance never exceeded chance level for any texture pair.

Julesz conjectures that particular texture pairs sharing certain statistical characteristics (e.g., power spectra) are discriminable at under 200 msec exposure because their elements approximate basic units for pattern recognition ("textons") in the visual system. If this theory is correct, then the demonstration of hemisphere differences in the ability to discriminate these texture pairs would suggest that higher-order cognitive operations can influence the most basic perceptual processes. It is also interesting to consider the direction of the hemisphere effect found: a left hemisphere superiority. In Julesz's theory, a change in local conspicuous features (textons) in a field being viewed results in a shift in use from the pre-attentive (or "ground") visual system, to the attentive (or "figural") visual system. A finding of left hemisphere superiority for texture discrimination thus supports previous work which describes the left hemisphere as a feature extractor, and the right as the hemisphere better able to extract information from the entire field--ground as well as figure. Replication and extension of these preliminary results is needed before the implications can be considered in greater detail.

190. APPARENT MOTION PERCEPTION BY COMMISSUROTOMY PATIENTS

Investigator: Jay J. Myers

Despite the supposed necessity of the forebrain commissures for transmission of visual perceptual information between the hemispheres, patients in whom these commissures have been surgically divided (to relieve intractable epilepsy) appear to retain some perceptual unity across the vertical meridian of their field of vision. Verbal reports from these subjects have demonstrated that their left hemisphere can crudely perceive large moving forms lateralized at least 15 degrees to the left of the visual midline (Trevarthen and Sperry, 1973). The subjects also apparently were able to interrelate such peripheral stimuli when presented to opposite sides of the midline. These findings were taken to support the existence of an extrageniculostriate visual system, involving midbrain structures, which is sensitive to movement and orientation in the visual periphery. Findings from tests of apparent motion perception in these subjects now suggest that this visual system may also convey to the cortex information about the position and perhaps also the movement and direction of stimuli in central vision.

Three complete commissurotomy subjects (LB, NG and RY) were tested for the perception of apparent motion within each visual hemifield and across the visual midline. A stroboscopic apparent motion effect was obtained by tachistoscopically flashing 2 one-half degree white dots against a black background for 20 msec each, with an interstimulus interval of 80-120 msec. These stimuli were separated by 3-4 degrees and were presented within 3 degrees of a central fixation mark. All three patients could, verbally or by pointing, correctly report apparent movement within either hemifield or across the midline. NG did, however, show some confusion of direction and perceived a discontinuity of movement at the midline while RY had difficulty perceiving motion across the midline in a right-to-left direction. In the absence of the commissures, midbrain structures thus seem to allow the unified perception of motion across the midline.

Reference:
Task Load and Response Mode Affects Dichotic Processing

Investigator: Polly J. Henninger

This study investigated the relative influence of task load and response mode on the right ear (left hemisphere) advantage for verbal stimuli in a right-handed commissurotomy patient (RY). It was conjectured that motor, cognitive and perceptual factors differ in the degree to which they require left hemisphere processing resources and, therefore, might differ in the extent to which they allow the expression of input to the left ear (right hemisphere). The study compared written, pointing and oral responses on a digit recognition test. Strings of increasing numbers of digits (one to four) were presented dichotically under each response condition at three levels of intensity: volume raised in the left ear (six dB louder), volume equal and volume raised in the right ear.

Under the written and pointing conditions, when the volume was biased to the left ear, the subject initially reported more of the digits presented to the left ear. As the number of digits increased, he reported more right ear digits than left. This suggests that as the material to be retained and processed increased, the cognitive factor overruled the perceptual one. However, in the oral condition, despite the intensity bias, the subject reported more of the right ear digits regardless of the task load. (When the volume to both ears was equal or louder in the right ear, the subject reported more of the right ear digits than left at all levels of task load and under all response conditions.)

The interaction between cognitive, perceptual and motor factors suggests that the right ear advantage for verbal stimuli is highly dependent upon the extent to which task factors utilize left hemisphere resources. The results indicate the need for careful analysis, in cerebral terms, of the motor requirements presented by the mode of response used. The progressive decline in left ear report as the number of digits presented increases, with no change in the physical characteristics of the stimulus or the motor requirements of the task, indicates that hemisphere differences reflect more than discriminations at the sensory or motor level as suggested by Bradshaw and Nettleton (1981) or differential sensitivity to frequency characteristics as proposed by Sergent (1982). These data suggest an independent, cognitive basis for asymmetries as well that is not based on stimulus qualities.

References:

Effects of Commissurotomy on Electrodermal Activities

Investigators: Evelyn L. Teng, Jeffrey J. Catania*, John C. Woolum

Five commissurotomy patients and six normal controls were individually tested under various conditions while the electrodermal activities (EDA) of their left and right hands were being recorded simultaneously. When subjects performed on tasks designed to engage preferentially either one or the other hemisphere, commissurotomy patients showed more divergent shifts in EDA levels between the two hands than the controls. In addition, patients also showed less phasic responses than the controls. On the other hand, commissurotomy had no apparent effect on the overall EDA level.

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LATERALITY OF SPATIAL DISCRIMINATIONS

Investigators: Charles R. Hamilton, Betty A. Vermeire

Last year we reported that the ability to discriminate lines of different slopes was done significantly better by the left hemisphere of split-brain monkeys than by the right (Biology 1982, No. 206). We decided to further check the idea that spatial discriminations were preferentially lateralized in monkeys by testing another spatial ability that is also lateralized in man, detecting the difference in position of dots within a visual frame of reference. Four discriminations, two between vertical differences in position and two between lateral displacement, were taught to each hemisphere of 16 split-brain monkeys. Eight monkeys were right-handed and eight were left-
handed; within these groups four were male and four were female. So far, 14 monkeys have completed the experiment. As with line orientation, the left hemisphere learned more easily, although the results are not quite significant (p <0.1). However, there was a significant correlation between the magnitude of lateralization of the discrimination and the degree of handedness of the monkeys such that the hemisphere opposite the preferred hand learned more readily (p <0.05), a result similar to that reported for discriminating between pairs of sequentially presented stimuli (Biology 1982, No. 208). It seems likely that a larger, significant hemispheric difference will be found with a more subtle discrimination; the separation of the dots in this experiment was almost 30% of the distance between the edges of the surrounding visual frame.

194. LATERALIZATION OF FACIAL EXPRESSION AND INDIVIDUAL RECOGNITION IN MONKEYS

Investigators: Betty A. Vermeire, Charles R. Hamilton

Facial recognition is widely regarded as a specialized ability of the right hemisphere in man. Our early work showed no hemispheric superiority in split-brain monkeys learning discriminations of monkey faces (Biology 1975, No. 154). However, recognition of the emotional content of facial expression is even more lateralized to the right hemisphere in man than is facial identification. Therefore, as we reported last year (Biology 1982, No. 207), our split-brain monkeys are now learning four discriminations based on differences in expression with the individual held constant and four discriminations based on identifying individuals with expression held constant. Overall, the 15 monkeys which have finished showed no significant advantage in learning with the left or right hemisphere. However, the correlation of dominance and handedness indices is almost significant (p <0.1), which suggests that there may be a tendency for the hemisphere opposite the preferred hand to learn more readily. When dominance on the four problems based on expressions and the four problems based on identity are compared, the difference is also nearly significant (p <0.1), in accord with our expectations from the results with human subjects. Although neither sex showed greater lateralization when compared over all eight problems, the eight female monkeys showed significantly better learning with their right hemispheres (p <0.01) on the four discriminations based on expression.

Nine of the monkeys have been tested for retention of the problems they learned and all remembered them well with either hemisphere. At present, there is an almost significant advantage for remembering with the right hemisphere (p <0.1). Seven monkeys have been tested for generalization of their performance to new exemplars in four of the discriminations. These monkeys performed well above chance with the novel stimuli, confirming that the expressions or individuals were discriminated according to the category intended and not simply learned as a series of independent stimuli. So far, there is no significant difference between the hemispheres in the percent correct responses made to the novel faces, but a cursory analysis of errors suggests the possibility that the two hemispheres may differ in the way they generalize to new faces. All these results suggest that the two hemispheres of split-brain monkeys may differentially process facial and emotional stimuli, but firm conclusions must wait until the entire design is completed.

195. VISUAL PREFERENCES OF SPLIT-BRAIN MONKEYS

Investigators: Betty A. Vermeire, Catherine K. Ifune*, Charles R. Hamilton

Previous work revealed that split-brain monkeys spent more time viewing interesting photographs with their left hemisphere than with their right, although no differential preferences correlated with stimulus content were observable (Biology 1980, No. 216). Two problems with this technique have now been overcome. First, the stationary pictorial stimuli have been replaced with action-filled videotapes composed of ten 2.5-minute segments of people, monkeys, zoo animals, and scenery. Second, the response measure has been broadened to include behaviors other than simply viewing. We observed that when freed from the constraints of an operant paradigm our monkeys displayed a wide variety of species-typical
responses such as lip-smacking, threatening, and "presenting" while viewing the videotapes. We can now look for characteristic differences in the way the two hemispheres behave while perceiving and processing meaningful stimuli by temporarily occluding either the right or left eye. With the support of a SURF fellowship, we are testing eight split-brain monkeys for hemispheric asymmetries in their responses to affective visual stimuli.

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196. INTERHEMISPHERIC TRANSFER OF DISCRIMINATIONS BASED ON MOVEMENT

Investigators: Charles R. Hamilton, Betty A. Vermeire

One of our research goals is to clarify the functions of different regions of cortex by determining the kinds and extent of information interhemispherically transferred by the commissural connections of the different regions (Biology 1978, No. 207). This approach complements anatomical and physiological studies by providing behavioral estimates of function but avoids many interpretative difficulties associated with using traditional brain lesions. We are studying discrimination of direction of movement because neurons well suited for this ability are conspicuous in areas deep in the superior temporal sulcus of monkeys and because we know where their commissural connections are located. Before evaluating with partial disconnection methods the participation of these areas in movement discrimination, we must determine the extent of subcortical transfer of movement. In previous work we found a small but significant amount of interhemispheric transfer in cats and monkeys with their forebrain commissures sectioned (Biology 1978, No. 208; 1974, No. 138). We could not then decide if the average savings of about 20% for relearning with the untrained eye represented transfer of specific information about the discrimination or represented some nonspecific or even artifactual advantage of training the second side after the first. We now have two results showing that specific sensory information crosses at subcortical levels.

Nineteen "standard" forebrain split monkeys that learned to discriminate expanding from contracting spirals with one eye have shown significant interhemispheric transfer; the average savings score when learning with the second eye was 27% (p <0.01). Five split-brain monkeys with additional section of the midbrain commissures have not shown significant interhemispheric transfer; their average savings score was -6%. Because nonspecific advantages should affect either group of monkeys equally when the second side is tested, we conclude that the savings score of the forebrain split group represents a real transfer of information. Furthermore, the midbrain commissures appear to be the route for this transfer.

A second problem based on direction of rotation of a ray-like pattern gave paradoxical results; the savings score for 10 forebrain-split monkeys was -18% (p <0.1), and was significantly different from transfer of the spirals (p <0.05). This negative transfer may represent another example of the paradoxical transfer found in monkeys with section of the optic chiasm when they learn to discriminate left-right mirror images (Biology 1972, No. 107). In the case of movement discriminations, however, the paradoxical transfer occurred via the midbrain commissures because the five monkeys with additional section of these commissures did not show significant savings, positive or negative. This residual transfer in forebrain split monkeys must be taken into account when evaluating interhemispheric transfer in partially split-brain monkeys.

197. INTEROCULAR TRANSFER OF TILT AFTEREFFECT IN PARTIALLY SPLIT-BRAIN MONKEYS

Investigators: Charles R. Hamilton, Betty A. Vermeire

The tilt aftereffect is an illusory tilt of an objectively vertical line that occurs after viewing a grating of straight lines tilted about 10° from vertical in the direction opposite to the induced tilt. This illusion is particularly interesting because normal human subjects who view the grating with one eye still see the illusion with the other eye while stereoblind humans usually do not. Because stereoblind monkeys and cats lack the normal complement of binocular neurons in V1 and V2, the deficit in interocular transfer is usually attributed to the loss of binocularity early in the
visual system. A direct test of this possibility is possible in monkeys because we can surgically vary the place where binocularity is first allowed and measure the degree of interocular transfer that is present. Transfer should be lost when two eyes are uncoupled at the place generating the aftereffect.

Last year we reported that our monkeys behaved as if they also perceived the tilt aftereffect (Biology 1982, No. 209). We have now tested interocular transfer of the aftereffect in three monkeys with the optic chiasm and cortical commissures, except for either the splenium of the corpus callosum or the anterior commissure, sectioned in the midline. The two monkeys with the intact splenium showed interocular transfer as expected because V1 and V2 still have binocular neurons along the vertical midline of the visual field. More surprisingly, the monkey with the intact anterior commissure also showed interocular transfer even though there was no binocularity left in the posterior visual areas. This implies that the inferotemporal areas are also capable of generating or sustaining the aftereffect. We are pursuing these findings with additional tests and animals and will compare these results to those from tests of stereopsis in the same animals.

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Summary: Our group is interested in the processing of information in higher visual areas of the macaque monkey. The cerebral cortex of the macaque contains a large number of distinct visual areas that collectively carry out many diverse aspects of visual perception and attention. We use a combination of physiological and anatomical techniques to identify visual areas, to trace the connections between them, and to ascertain the functional properties of their constituent neurons.

A total of 13 visual areas have been identified to date in the macaque, and there is good reason to anticipate that additional areas will be discovered in the near future. These areas form a large mosaic occupying more than half of the entire cerebral cortex. The connections among the various areas are quite complex, as each area typically has multiple inputs and multiple outputs. Fortunately, though, we have recently found that the pattern of connectivity can be used to delineate an overall

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hierarchy of cortical areas. In this hierarchical scheme, each area occupies a well-defined position, or rank, with respect to the other visual areas, as shown in the figure below.

The 13 visual areas are arranged in six hierarchical levels. V1, the primary visual cortex, is at the bottom of the hierarchy, and areas in the temporal, parietal, and frontal lobes are at the uppermost levels. A characteristic feature of this system is that connections between areas are generally organized as reciprocal pairs. For example, area V1 contains cells whose axons project to area V2, and V2 contains cells that project back to V1. The assignment of which area is higher and which area is lower for any interconnected pair is based on characteristic differences in the laminar organization of inputs and outputs. Ascending (forward going) pathways originate from cells in upper layers of the cortex and terminate mainly in the middle layer (layer IV). Descending (feedback) pathways originate from cells in both lower and upper layers and terminate outside layer IV.

Physiological recordings from neurons in different visual areas have suggested that there are separate functional streams for the processing of information about visual motion on the one hand, and form, pattern and color on the other hand. The motion pathway includes area MT, whose properties we have studied in detail in previous years. Currently, our major interest is in studying areas involved in the pathway for analyzing form and color.
A separate facet of our research program concerns the development and plasticity of the mammalian neuromuscular junction. Muscle fibers at birth have multiple synaptic inputs, and the excess inputs are selectively eliminated during early postnatal maturation. Around the same time, muscle fibers differentiate into separate populations of slowly and rapidly contracting fibers. Interestingly, we have found that fast and slow motor neurons have established selective connections with the appropriate muscle fiber types already at birth, well before the period of wholesale synapse elimination is under way.

198. FUNCTIONAL PROPERTIES OF SINGLE CELLS IN THE VENTRAL POSTERIOR AREA (VP) AND AREA V2 OF THE MACAQUE

Investigators: Andreas Burkhalter, David C. Van Essen

As part of our long-term goal of ascertaining the degree of functional specialization in different cortical areas, we have studied the physiological properties of neurons in two visual areas, V2 and VP, in the ventral portion of extrastriate cortex in the macaque. Neurons were tested for their selectivity to stimulus shape, orientation, direction and speed of motion, binocular disparity, and color. Subsequent histological analysis, using the pattern of callosal inputs as a marker for areal boundaries, indicated that 96 of the cells in our sample were in VP and 56 were in V2.

The most interesting result to emerge from this study concerns the incidence of color selectivity in VP and V2. We found that cells did not fall into natural groupings of highly color selective on the one hand or completely non-selective on the other hand. We therefore devised quantitative measures which compare the relative responses to different wavelengths. Using this approach, we found that the majority of cells in VP (60%) and V2 (64%) were color selective in the sense of responding at least three times better to the optimal color than to the least effective color.

The majority of cells in VP and V2 were strongly selective for the orientation of elongated stimuli, and about half were disparity selective and thus able to signal information about depth in space. Only a small minority of cells were selective for the direction of stimulus motion. The high percentage of color cells and low percentage of direction-selective cells contrasts sharply with the properties of cells in area MT, where the reverse pattern is found. Thus, these results support the notion that the pathway for the analysis of form and color is largely separate from that for analysis of motion.

Studies in other laboratories have shown that in striate cortex, color selective cells are preferentially located in clusters (within cytochrome oxidase-rich "blobs"), and that these cells generally lack orientation selectivity. In VP we found that the color selective cells are also spatially organized, with cells of similar color preference clustered together. Interestingly, we found that the great majority of color cells in VP were also orientation selective, suggesting that the convergence of information about form and color may be an important aspect of function in higher visual areas.

199. THE CONNECTIONS OF THE VENTRAL POSTERIOR AREA (VP) IN THE MACAQUE MONKEY

Investigators: Andreas Burkhalter, David C. Van Essen

In the preceding report we described several interesting aspects of functional processing in neurons of area VP in extrastriate cortex of the macaque. In order to ascertain where the inputs to this area originate and where its outputs are distributed, we have made injections of anatomical tracers into identified regions within VP. By using a combination of a retrograde tracer, horseradish peroxidase (HRP), and an anterograde tracer (3H-proline), it was possible to identify both input and output connections in each experiment. In addition, the corpus callosum was cut, so that the pattern of degenerating terminals could be used to identify the boundaries of several visual areas.

Our results indicate that VP has reciprocal connections with at least six cortical areas. On the basis of information about the cortical layers in which pathways originated and terminated, it was possible to designate each connection as ascending or descending, as discussed in the Summary. The
only ascending input to VP is from V2. We found no evidence for a direct connection between VP and V1. This observation confirms an important asymmetry in the connections of V1 that we had previously discovered when making tracer injections into V1.

The ascending outputs of VP are distributed to several topographically organized extrastriate areas, including MT, V3A, and an as yet unnamed area anterior to VP which in turn is known to project to inferotemporal cortex. In addition, there are projections to cortex in the parietal lobe and to several subcortical nuclei.

It is of interest to compare the projections of VP, which adjoins ventral V2 and represents the upper part of the visual field, with those of V3, which adjoins dorsal V2 and represents the lower part of the visual field. The connections of VP identified in the present study differ in several important respects from those reported by Zeki after lesions in V3. This strengthens the argument that information from upper and lower parts of the visual field is processed in distinctly different fashion in extrastriate cortex of primates.

200. FUNCTIONAL PROPERTIES OF NEURONS IN AREA V4 OF MACAQUE MONKEY EXTRASTRIATE CORTEX

Investigators: Daniel J. Felleman, David C. Van Essen

Area V4 is a region of extrastriate cortex of macaque monkeys first recognized more than a decade ago on the basis of its strong inputs from V2. V4 has a major projection to the temporal lobe, a region believed to play an important role in the analysis of pattern, color and form in the visual world. Early investigations by Zeki of the functional properties of neurons in V4 suggested that nearly all cells were selective for the wavelength of stimuli. However, more recent reports from several laboratories have estimated the proportion of color-selective neurons in V4 to be much less, perhaps as low as 20-35%. Unfortunately, there has been considerable ambiguity in the criteria used for classification of cells as color selective in these studies and thus, it is not unreasonable to expect some disagreement in the population estimates.

Recently, this laboratory has developed a quantitative technique to evaluate color selectivity of cortical neurons on the basis of the differential responses to monochromatic and white stimuli matched in brightness. This technique provides two metrics which together provide a standard way of evaluating the influence of wavelength on neural response. We are currently using this approach to reinvestigate the color selectivity of cells in V4. In addition, we are interested in determining other receptive field properties of V4 neurons such as the selectivity for orientation, direction, velocity, binocular disparity, and stimulus form. To date, we have recorded from 40 neurons in area V4 and have characterized their selectivities for several of the aforementioned stimulus parameters. Although the sample is still small, it is apparent that the majority of cells in V4 are selective for stimulus wavelength. In addition to their color selectivity, most V4 cells are orientation selective and some are selective for binocular disparity. Thus, depth and color are not specifically analyzed in any one cortical area. Finally, the distribution of preferred velocities in V4 is remarkably homogeneous, with almost all cells preferring movement of 8-16°/second. This is in marked contrast to area MT where cells have preferred velocities extending over two orders of magnitude.

Future research will expand the data base of neurons in V4 and will concentrate on investigating the influence of background illumination conditions on the color selectivity of these neurons. We hope this will lead to a better understanding of the physiological mechanisms underlying the perceptual phenomena of color contrast and color constancy.

201. CONNECTIONS OF AREA V4 OF MACAQUE MONKEY VISUAL CORTEX

Investigators: Daniel J. Felleman, David C. Van Essen

V4 is a visual area that is clearly distinguishable from neighboring cortical areas (MT on one side and V3A on the other) by several anatomical and physiological criteria, but whose internal organization is surprisingly complex. While we have begun to understand the functional properties of neurons in this area, much remains to be determined about its inputs from and outputs to other cortical areas and about the complexities of its
internal organization. We are addressing these issues by making injections of several tracer substances, including horseradish peroxidase (HRP) and 3H-proline.

Previous work from this laboratory has described a hierarchy for visual cortical areas in the macaque based on the laminar distribution of cell bodies and axon terminals associated with each projection from one area to another. Using this approach, it is now possible to more clearly assign the hierarchical position of V4 and to describe some additional anatomical connections. V4 receives ascending (feedforward) connections from areas V2, V3, and V3A and provides feedback (descending) projections to these areas as well as a weak projection to V1. The major ascending outputs of V4 are to the temporal lobe. Our preliminary results suggest that there are at least two targets in the temporal lobe, one termed IT (inferotemporal cortex) and the other situated more ventral and medial in the region known as TF-TH on the basis of cytoarchitectonic studies. It appears that both of these areas provide feedback projections to V4. V4 also has connections with area MT which appear to be of the feedback or intermediate variety. A projection from V4 to cortex in the parietal lobe that has been reported in other studies was found in only one of the two experiments done so far.

The pattern of local connections within V4 suggests that it may contain distinct, reciprocally connected posterior and anterior subdivisions. Further studies will be necessary to determine whether these regions should be considered as separate visual areas occupying different levels of the cortical hierarchy.

202. PATCHES OF STRIATE CORTEX WHICH PROJECT TO V2 ARE COINCIDENT WITH CYTOCHROME OXIDASE "BLOBS"

Investigators: Daniel J. Felleman, David C. Van Essen

One area of neurobiology that has recently received a great deal of attention concerns the modular organization of cerebral cortex. What was once thought to be a relatively homogeneous structure in the horizontal dimension has proven to be highly complex when examined with modern anatomical and physiological techniques. Recently, several groups have examined the distribution of the mitochondrial enzyme cytochrome oxidase in primate striate cortex and have revealed an array of patches of increased enzyme activity, mainly in the superficial layers of cortex. These "blobs" are roughly 200 µm across and appear in rows spaced approximately 300-500 µm apart. Investigations of the physiology of neurons contained within the "blobs" have indicated that many cells lack the orientation selectivity characteristic of superficial striate cortex and furthermore, that many are selective for the wavelength of stimuli.

The major output of striate cortex (V1) is to a large adjoining area, V2. In V2, regions of increased cytochrome oxidase activity appear as tangential stripes running approximately perpendicular to the striate border. We are using anatomical techniques to study the relationship between the cytochrome oxidase patterns in V1 and V2. Following a small injection of the retrograde label horseradish peroxidase (HRP) into V2 and the subsequent transport to cell bodies in V1, a large portion of V1 and V2 is removed, flattened and sectioned parallel to the cortical surface. Sections are then reacted for the presence of HRP. The result from one successful experiment to date was striking. Retrogradely filled cell bodies were found in the superficial layers of V1 (layers II and III) and occurred in patches of 2-10 cells in any given section. When sections were aligned to span the depth of cortex, these cell clusters aligned into cylinders extending through layers II and III. Adjacent histological sections were reacted for the demonstration of cytochrome oxidase and carefully aligned with the HRP sections using the numerous blood vessels and surface landmarks. The HRP filled cells were coincident with the patches of increased cytochrome oxidase activity. Thus it appears that a restricted subset of V1 neurons, apparently lacking orientation selectivity and possessing color selectivity, provides input to restricted portions of V2. Preliminary evidence suggests that adjoining regions of V2 receive input from "non-blob" regions of V1, since large injections of HRP into V2 label a continuous zone of V1. Further work will be necessary to elucidate the apparent modular organization of V2 and to
determine how precise the relationship is between cells in the cytochrome oxidase "blobs" in V1 and "stripes" in V2.

203. COMPUTERIZED TWO-DIMENSIONAL MAPS OF THE CEREBRAL CORTEX

Investigators: George J. Carmean, David C. Van Essen

The convolutions formed by the gyri and sulci of the cerebral cortex pose difficulties for the study of the functional organization of this structure. Fortunately, these convolutions arise through folding of an initially smooth cortical surface during development; the resultant surface does not have a high degree of intrinsic, or three-dimensional curvature. This makes it feasible to generate two-dimensional maps of the unfolded cerebral cortex, upon which anatomical and physiological data can be represented (Van Essen and Maunsell, 1980).

At present, such cortical maps are produced through manual procedures which involve manipulation of information obtained from histological sections of the brain. While these manual procedures have demonstrated the feasibility of this approach, they are both tedious and subjective, which limits their utility and reproducibility. In order to overcome these difficulties, we are attempting to develop a set of algorithms which will enable a laboratory computer to produce two-dimensional cortical maps more efficiently and objectively than is currently possible.

These algorithms will utilize information about the three-dimensional cortical surface obtained from histological sections of the brain to generate a two-dimensional approximate representation of the cerebral cortex within the computer. In this representation, each point is coupled to neighboring points by elastic elements whose dimensions can be compared with those of the corresponding line segments on the cortical surface. The algorithms seek to optimize the dimensions of all elements by iterative adjustment of the local geometry of the elastic sheet. Each iteration leads to a incremental relaxation of the sheet, ultimately producing a final conformation which maintains topology, preserves area, and minimizes distortion. This final conformation is the two-dimensional map of the cortical surface.

Thus far, these relaxation algorithms have been successfully used to map simple surfaces in an evaluation of their performance. We hope to test their ability to map more complex surfaces, and ultimately the primate cerebral cortex, within the coming year.

Reference:

204. SELECTIVE INNERVATION OF MUSCLE FIBER TYPES IN A DEVELOPMENTALLY POLYINNERVATED MUSCLE

Investigators: Herman Gordon, David C. Van Essen

Mammalian skeletal muscles pass through a stage of early postnatal development when each muscle fiber is transiently polyinnervated by several different motor neurons. During the period of polyinnervation, fibers within a single muscle differentiate into slow and fast twitch types as distinguished by their histochemical profiles. Do motor neurons preferentially innervate one or another type of muscle fiber? We addressed this question by eliciting muscle twitches in response to stimulation of single motor axons to the rabbit soleus shortly after birth, a period of three to fourfold polyinnervation. Twitches with distinctly different time courses were found, indicating a preferential innervation of particular muscle fibers by individual motor neurons. In fact, the diversity in times to peak of twitches is as marked in a neonatal polyinnervated muscle as it is ten days later once elimination of the redundant synapses has occurred. At the extremes of the spectrum, it seems likely that motor units contain predominantly either fast twitch or slow twitch muscle fibers. Thus, at least some motor neurons in this muscle can be typed according to the muscle fibers which they innervate, and motor neurons of a particular type share end plates preferentially with other motor neurons of the same type.

How could selective innervation of specific muscle fiber types by particular motor neuron types have come about during normal development? It is interesting to consider several possibilities. First, there might be a chemosaffinity, of the type
suggested by Roger Sperry, such that fast motor neurons bear surface receptors for fast muscle fiber markers, and similar situations would obtain for the other muscle fiber and motor neuron types. During development, motor axons would actively search for appropriate synaptic sites. Second, motor axons might instead show affinity for other motor axons of like type, and preferentially fasciculate together down to the same end plates. Through inductive factors or activity patterns coordinated among the several like inputs, each muscle fiber would then acquire the type defined by its inputs. Third, there is evidence that muscle fibers are born in at least two waves of myogenesis in which the first fibers born become predominantly slow twitch and the second fibers become predominantly fast twitch. One could imagine a well-choreographed timing in which slow motor neurons are the first to grow into a muscle and are able to extensively polyinnervate the initial group of myotubes. Then, in a second wave of innervation, fast motor neurons would grow into the muscle and find a largely separate population of fibers available for polyinnervation. It may be possible to test these possibilities using anatomical techniques.

**PUBLICATIONS**


NEUROGENETICS

Seymour Benzer
Summary: Our activities are directed at mechanisms underlying the development and function of the nervous system, using the fruit fly, Drosophila, in which connections between genetics, molecular biology, physiology and behavior can be traced.

Monoclonal antibodies offer highly selective markers for identifying surface molecules that distinguish neurons and other nervous system components from one another. By injection of Drosophila brains into mice and subsequent hybridization of the host’s activated spleen cells with a myeloma cell line, we have generated a large collection of hybridomas that produce monoclonal antibodies. These are being used to study the chemical architecture of the nervous system, the developmental appearance of each antigen, and the genes involved.

Special attention is directed at the development of the Drosophila compound eye, which involves formation of photoreceptor cell clusters in a highly precise array, and at antigenic homologies between the Drosophila and human nervous systems. Recombinant DNA technology is being employed for the isolation and cloning of relevant genes.

205. SEQUENTIAL APPEARANCE OF RETINAL ANTIGENS, REVEALED BY MONOCLONAL ANTIBODIES

Investigators: Stephen L. Zipursky, Tadmiri R. Venkatesh, Seymour Benzer

The compound eye of Drosophila melanogaster is a precise, repetitive pattern comprising about 800 hexagonal units called ommatidia. Each ommatidium contains a lens, a crystalline cone, eight photoreceptor cells, four cone cells which secrete the material in the cone and lens, a sheath of pigment cells and a hair-nerve group of four cells. The framework of this pattern is established in the eye-antennal disc during the third larval instar. A wave of morphogenesis, marked by a groove (the morphogenetic furrow [MF]) sweeps anteriorly across the disc, leaving in its wake clusters of photoreceptor cells. Cells between clusters give rise to the remaining cells of each ommatidium. Genetic mosaic studies rule out strict cell lineage relationships between cells of a single ommatidium. Furthermore, there does not appear to be a precursor cell population which gives rise to a single class of ommatidial cells. Thus, a cell's identity appears to be determined by its position within the developing array (Ready et al., 1976).

We have used monoclonal antibodies (MAbs) to identify specific molecules which appear during retinal development. MAbs highlight pattern elements that are not readily observable by conventional techniques, providing useful reagents for further characterizing morphogenesis in normal and mutant flies. Furthermore, molecules whose appearance is correlated with important morphogenetic events can be purified with the aid of their respective MAbs.

MAb3E1 recognizes an antigen in a fibrous meshwork concentrated within the morphogenetic furrow and anterior to it. As development proceeds in the third instar, the differentiated posterior region of the eye disc becomes depleted of this antigen. Immediately posterior to the furrow, MAb6D6 recognizes cells which form a square array. This MAb stains cone cells in the adult eye. Each square encloses a cluster of photoreceptor cells. The axons of these cells are stained by MAb22C10 posterior to the morphogenetic furrow. This MAb also stains other nerves, from early embryo through to adult. MAb24B10 appears further behind the furrow, corresponding to a later stage of development. This MAb is photoreceptor cell-specific; it stains receptor cells and their axons in the larval photosensitive organ, the ocellus and the compound eye. Later, during mid-pupal development, MAb21A6 stains the junctions between photoreceptor cells.
At still later stages, MAb28H9 stains the rhabdomeres of the photoreceptor cells, and MAb3F12 stains the lens. In addition to using MAbs in the definition of a molecular genetic program of retinula cell development, we hope to use them to further characterize mutations which affect eye morphology. For instance, Lebovitz and Ready (1982) have shown that eye discs of the glass mutant lack response to one of their MAbs that does stain normal discs. For our set of MAbs, MAb22C10 does stain the array of axonal fibers in the eye disc of the mutant gl3, whereas MAbs 24810, 21A6 and 28H9 show no staining in the larval, pupal or adult eye. Thus, mutations can be used to dissect the sequence of molecular events during development of the neural array.

Reference:

206. MONOCLONAL ANTIBODIES USED TO PURIFY MOLECULES IN PHOTORECEPTOR CELL DEVELOPMENT
Investigators: Tadmiri R. Venkatesh, Stephen L. Zipursky, Seymour Benzer

The development of the photoreceptor cells of the Drosophila compound eye provides a model system for studying the contributions of genes to neuronal differentiation. In addition to common features of neurogenesis, such as axonogenesis and synaptogenesis, these cells acquire phototransduction capability.

We have used monoclonal antibodies to identify polypeptides which appear at different stages of photoreceptor cell development, as a step toward identifying the genes expressed. By studying the expression, organization and function of these genes, we may gain insight into the molecular events which determine the developmental pathway. By using immunoaffinity columns, four antibodies which define antigens appearing at different stages of development have been purified.

1) MAb22C10 stains the axons, including those of protophotoreceptor cell clusters behind the morphogenetic furrow. The antigen recognized by their MAb has a molecular weight of 220,000 and appears early in neural differentiation.

2) MAb24810 is specific to an antigen that occurs in photoreceptor cells only, appearing during late third instar far behind the morphogenetic furrow. It has a molecular weight of 160,000.

3) MAb21A6 reveals an antigen that first appears in the mid-pupal stage, between the developing photoreceptor cells. It also stains the bases of sensory hairs in the antenna. This antigen has a molecular weight of 250,000.

4) MAb28H9 stains the rhabdomeres themselves, which begin to appear in the late pupa. Preliminary biochemical analysis indicates that it has a molecular weight of 160,000.

207. N-TERMINAL AMINO ACID SEQUENCE OF A DROSOPHILA RETINAL ANTIGEN
Investigators: Tadmiri R. Venkatesh, Stephen L. Zipursky, David B. Teplow, Seymour Benzer

MAbs provide a unique opportunity to identify polypeptides which show cellular specificity within the nervous system. In order to gain an understanding of the roles these proteins play in neural development, it is desirable to study the biochemical properties of the purified proteins and to define the genes which encode them. Accordingly, we have used several MAbs, covalently attached to Sepharose 4B columns, to purify the corresponding neural antigens by chromatography of Drosophila head extracts. With respect to our interest in retinal development, we have purified, to near homogeneity, the antigens recognized by MAb22C10 and MAb24810, both of which appear on photoreceptor cells during the larval third instar.

Ag24810 is a membrane-associated, high molecular weight polypeptide of molecular weight 160,000. It appears to be as highly glycosylated as the serum protein fetuin (which has 8% sugar by weight), as determined by PAS staining of comparable amounts of the proteins in SDS-PAGE. We have confirmed the retinal specificity of this antigen in several ways. In two-dimensional gels of retina homogenates, it produced a single spot that is known to occur in homogenates made from whole heads but not from retina-less heads (Hotta, 1979). Purified 24B10 antigen inhibited staining.
of frozen sections of retina by MAb24B10. When injected into rabbits, it produced a retina-specific antiserum. Using a solid phase radioimmunoassay with $^{125}$I-labeled antigen 24B10, we estimated that the antigen comprises 0.1-0.25% of total head protein. Thus, the antigen appears to account for as much as 1-5% of the photoreceptor cell protein.

As a step towards identifying the gene encoding this molecule, we have determined 19 of the first 21 amino acids at its N-terminus. This required only 4.4 µg of protein. Two segments of the sequence have been selected, and the corresponding probes have been synthesized. These probes are currently being used to screen Drosophila DNA libraries to isolate the gene encoding the antigen.

Reference:

208. ANTIGENIC HOMOLOGIES BETWEEN HUMAN AND DROSOPHILA BRAIN

Investigators: Carol A. Miller, Seymour Benzer

Monoclonal antibodies provide a new dimension to neuroanatomy, revealing hitherto unsuspected subsystems identifiable by their molecular specificities. While there has been some application of monoclonal antibodies to the human nervous system, relatively little has been done as yet, partly due to the system's complexity and the difficulty of obtaining appropriate tissue.

We have found that there are many cross reactions between Drosophila and human tissues (Miller and Benzer, 1983). A panel of 146 antibodies, obtained with Drosophila melanogaster head tissues as primary immunogen, was tested against various human CNS elements. Sites examined included spinal cord, cerebellum, hippocampus, visual cortex, and optic nerve. Non-nervous tissues also tested were liver and lymph node.

Approximately 50% of the antibodies reacted with one or more sites in the human CNS, identifying regional, cell class and subcellular antigens. Some recognized neuronal, glial or axonal subsets. In contrast, there were few cross reactions with liver and lymph nodes.

The tissue distributions of these antigens in the two species have been compared, and the molecular homologies examined with the Western electroblot technique.

In addition to providing new markers for the human nervous system, the molecular characteristics of these antigens are of interest in respect to their evolutionary conservation and their specific functions in development, behavior and disease.

Reference:

209. ACETYLCHOLINE-INDUCED SINGLE CHANNEL CURRENTS IN DISASSOCIATED DROSOPHILA NEURONS

Investigators: Mark A. Tanouye, Chun-Fang Wu*, Steven H. Young**

Current flowing through an individual ion channel can be measured in small patches of nerve or muscle membrane (Hamill et al., 1981). This method may be particularly useful in analyzing ion channels with functional features altered by mutation (Biology 1982, No. 229). To this end, we have begun to examine single channel currents in normal Drosophila neurons. Subsequent studies will involve excitability mutants. The Drosophila neurons recorded from were type 1 and type 3 cells dissociated from the isolated nervous systems of third instar larvae and cultured in Schneider's tissue culture medium (Wu et al., 1983). Conventional patch recording methods were employed (Hamill et al., 1981). In brief, a small-diameter (1 µm i.d.) glass micropipette was used to electrically isolate and voltage clamp a small membrane patch on intact cells. Several ionic channel classes were observed when pipette-to-membrane seal resistances were greater than 10 G-Ohm. In this report, we describe single channel currents activated by the neurotransmitter acetylcholine (ACh).

ACh-induced currents were observed when the patch electrode was filled with saline containing ACh (10-100 nM). The currents persisted when the saline in the bath and electrode contained 3 µM tetrodotoxin, 20 mM tetraethylammonium, 5 mM 4-aminopyridine, and 1 mM cadmium. Surprisingly, the currents were not blocked when the cells were
pre-incubated with 50 µg/ml α-bungarotoxin, which blocks ACh receptor currents in vertebrate skeletal muscle. Channels tended to be active in bursts or clusters of activity. The frequency of channel opening was strongly dependent on the concentration of ACh in the pipette. At 20°C the mean channel open time was 2.0 to 3.5 msec and showed little voltage dependency. The current-voltage relation of the single channel currents was tested in the range of -80 to +40 mV in different patches and was found to be roughly linear. The apparent single channel conductance was approximately 9 to 20 pS. The reversal potential was +5 to +15 mV above the apparent resting potential.

References:

210. CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE PHOSPHODIESTERASE AND ITS ROLE IN LEARNING IN DROSOPHILA

Investigator: Sandra L. Shotwell

Drosophila carrying the X-linked mutation dunce (dnc) showed poor learning in a negative reinforcement olfactory conditioning paradigm (Dudai et al., 1976). More recently, dnc flies were shown to have reduced activity for one of two cAMP phosphodiesterases (PDEs) present in normal flies, PDE II, whereas PDE form I was unaffected (Byers et al., 1981). A microassay technique was described (Shotwell, 1983) that allowed the separate measurement of PDE I and PDE II in crude extracts, based on specific inhibition of PDE I [3H]cAMP hydrolysis by cGMP. Using this technique, PDE II was shown to occur normally at high specific activity in the nervous system, consistent with the hypothesis that this enzyme plays a role in neuronal function. Reduced PDE II activity correlated with poor learning in dnc flies at three developmental stages (first and third instar larva, and adult), as well as in response to genetic modification of dnc gene activity. Biochemical and genetic experiments failed to reveal any abnormal regulation of PDE II in dnc. The specific activity of PDE II was shown to correlate in a one-to-one fashion with the level of normal dnc gene activity at five different doses of dnc+ (Shotwell, 1983). These results support the hypothesis that PDE II represents the primary product of the dnc gene, indicating a role for this enzyme in Drosophila learning.

References:

211. DEFECTIVE CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE PHOSPHODIESTERASE IN THE DROSOPHILA MEMORY MUTANT dunce

Investigator: Lawrence M. Kauvar

A detailed characterization of the cyclic nucleotide phosphodiesterases (PDEs) from normal Drosophila melanogaster was made (Kauvar, 1982), including purification of the two major enzymes to near homogeneity. A third, more labile phosphodiesterase also was identified in crude homogenates. The total activity per fly of one of these three enzymes, PDE-II, is strongly influenced by the dnc locus. Two independently derived dnc mutants produce variants of PDE-II with modified intrinsic properties: a marked decrease of thermal stability in dnc1 and a tenfold increase in the Michaelis kinetic constant in dnc2. These defects, which persisted in purified preparations of PDE-II, were mapped genetically to dnc. The results support the identification of dnc as the structural locus for PDE-II. The tight connection between the dnc gene and the PDE-II enzyme indicates that defective cyclic adenosine 3':5'-monophosphate metabolism is the primary lesion which leads to failure of dnc flies to learn in the olfactory associative conditioning paradigm of Quinn et al. (1974).

References:
PUBLICATIONS


DEVELOPMENTAL GENETICS

Edward B. Lewis
The bithorax gene complex in Drosophila continues to occupy our attention since it promises to show us how genes are regulated not only very early in development but during all stages thereof and in all tissues of the body. Our report this year especially concerns three unusual new cis-dominant mutants, Transabdominal, Superabdominal and Cephalotergite, each of which presumably represents a gain, rather than loss, of function. This work is a part of our larger goal of saturating the bithorax region with mutants, especially of the cis-dominant gain-of-function type, in order to try to identify as many as possible of the vast number of genetic functions that seem to be a part of the bithorax complex and that are evidently necessary in order to achieve the highly evolved states of segmental differentiation found in the thorax and abdomen of the fly. At the same time these studies should provide the necessary lesions in the DNA of this complex that are vital for the molecular analysis of the complex now being so vigorously pursued in the laboratories of D. Hogness (Stanford), W. Bender (Harvard Medical School) and M. Akam (Cambridge University).

212. TRANSABDOMINAL: A NEW DOMINANT MUTANT IN THE BITHORAX GENE COMPLEX

Investigators: E. B. Lewis, Josephine Macenka

A new dominant mutant, Transabdominal (Tab), produces stripes of abdominal tissue on the top of the thorax. The mutant was induced with X rays and is associated with a small inversion having one breakage point within the bithorax complex in 89E and another in 90E. Scanning electron microscope studies indicate that the thoracic stripes of Tab flies correspond to abdominal tissue that is normally found only on the dorsal surface, or tergite, of the sixth abdominal segment (A6). The latter segment and the fifth (A5), as well, are black in males but only banded with black pigment in the female. As a result, Tab flies show pronounced sexual dimorphism not only in the abdomen but in the thorax as well. Although invariant, the pattern of thoracic striping in Tab flies is complex. Whereas in the abdomen of wild-type or Tab flies the tergite of A6 is a broad band of tissue running transversely, in the thorax of Tab flies the presumptive A6 tissue consists of two sets of narrow bands running longitudinally. One of these sets consists of two bands symmetrically located close to, and on either side of, the midline. These bands extend from the neck region backwards about one-third the length of the thorax; they are black in the male but devoid of pigment in the female. The second set of bands consists of two bands symmetrically located about half way between the midline and the lateral edge of the notum; these bands extend somewhat farther back and end in a raised vortex just lateral to the dorso-central region of the thorax. The second set of bands forms a wider streak of black-pigmented tissue in the male than it does in the female, and in both sexes the bands are transversely interrupted in their pigmentation to form two separate sections instead of continuous stripes. The exact boundaries between thoracic and presumptive abdominal tissue are therefore not so clear in the second set of stripes as they are in the first.

Tab is lethal when opposite a chromosome deficient for the bithorax complex (DF-P9) and expresses an embryonic phenotype that suggests that A8 is converted towards A6 or A7. The associated inversion breakage point in 89E may therefore represent a new lesion in the extreme distal portion of the complex.

The extremely bizarre patterning of ectopic abdominal tissue in Tab suggests that this mutant
will be a useful tool for studying not only differentiation of abdominal versus thoracic tissue but also the mechanism of gene regulation within the bithorax gene complex.

213. POLAR POSITION EFFECT WITHIN THE BITHORAX COMPLEX

Investigators: E. B. Lewis, Rollin H. Baker, Josephine Macenka

The bithorax gene complex in Drosophila is polarized in the sense that a given mutant tends to reduce the functioning of one or more genes located distally, but not proximally, to that mutant. The complex thus exhibits a functional polarity or polar position effect (PPE). To illustrate, consider bithorax-3 (bx3) and postbithorax (pbx). The former mutant transforms the anterior, and the latter, the posterior, portions of the third thoracic segment (T3) into structures resembling the corresponding portions of the second thoracic segment (T2). In the trans configuration, bx3/pbx, there is a weak pbx effect (enlarged posterior portion of the haltere) but no bx effect; that is, no reduction in function of the bx+ gene proximal to pbx is detectable. The cis heterozygote, bx3 pbx/+, is wild type, as expected in this type of position effect.

In bacteria, such position effects in operons have been shown to coincide with the direction of transcription. Although the evidence is incomplete, unpublished molecular studies of D. Hogness and his collaborators (personal communication) indicate that the direction of transcription for many messages in the proximal portion of the complex is not proximal to distal, but the reverse. Furthermore, Bender et al. (1983) show that a relatively immense distance separates bx3 from pbx; namely, over 50 kilobases (kb) and of course the intervening region includes the functions of two other major genes of the complex, Ultrabithorax (Ubx) and bithoraxoid (bxd).

We are concentrating on trying to determine how universal and how invariant are the rules governing polarized position effects within the bithorax complex. In particular, we have been investigating an apparent exception to the rule that the polarity proceeds always in one direction. It has been long known that two mutants, bx and pbx interact in the trans configuration, bx/pbx, to give a moderate pbx phenotype. In this case the bx mutant, when homozygous or opposite a deficiency, shows a partial conversion of the first abdominal segment (A1) into a thoracic one; combined with this phenotype is a moderate pbx phenotype. The failure of bx/pbx animals to show a trace of the property of bx to effect a thoracic modification of A1 is in keeping with the rule that the functioning of the bx+ gene located proximally, and in cis, to pbx is not noticeably reduced. On the other hand, if pbx is tested against a third chromosome lacking either all of the bithorax complex, the Df-P9 deficiency, or, as in Df-P10, lacking the region from abx to pbx, inclusive, the resultant hemizygote, pbx/0, now shows a variable bx phenotype in the sense of having a variably reduced tergite in A1 and a thoracic modification of the spiracle of that segment. It is as if pbx exerts a polar effect on the bx+ gene which adjoins it proximally in cis. This exception to the rule already outlined above for polar position effects in the complex may be only an apparent one that results from special peculiarities associated with partially haploid genotypes of the pbx/0 type. Thus, in pbx/0 a gene such as Ubx+ is present in only one dose instead of the usual two doses found in the bx/pbx heterozygote. This reduction in dosage of Ubx+ could account for the bx phenotype in the pbx/0 case, since Ubx+ function is known to be required during embryonic development of not only T3 but of A1 (and other abdominal segments) as well.

To test the possibility of an effect of dosage of Ubx+, we have made use of a previously constructed chromosome described in detail elsewhere (Lewis, 1978), which carries in the left arm of the third chromosome a duplication, Dp-100, that includes only the most proximal portion of the complex from abx+ to Ubx+, inclusive, and in the right arm, the deficiency, Df-P10. This derived chromosome is effectively deficient for only the bx and pbx genes of the complex and can be symbolized, bx+pbx+. When this chromosome is combined with pbx, the resultant bx+pbx+/bx+ pbx genotype still has a very strong pbx phenotype but has a
wild-type first abdominal segment. We conclude that the exception to the polarity rule in the case of the pbx/O genotype is only an apparent one and arises from the failure to maintain in that genotype two doses of Ubx+. A corollary is that two doses of Ubx+ are critical for normal differentiation of A1 when pbx function is reduced or absent.

References:

214. TRANSVECTION IN TANDEM DUPLICATIONS
Investigators: E. B. Lewis, David J. Baker, Josephine Macenka

Transvection is an unusual type of position effect that appears to require gene action at a relatively great distance (Lewis, 1954). Consider, for example, the trans heterozygote, bxd+/pbx2. Even though the wild-type alleles of the bxd and pbx2 mutants are relatively far apart since they are in opposite chromosomes, they have been shown to cooperate effectively with one another, producing a much more nearly wild-type phenotype when the chromosomes are allowed to pair normally than when any of certain gross, pairing-disrupting, or "transvecting" rearrangements is present in either the bxd or pbx2 chromosome (see the previous abstract for a description of the bxd and pbx phenotypes) (Lewis, 1982).

The mechanism underlying the transvection phenomenon is unknown and apparently involves new principles of gene regulation at the chromosome level. We are attempting to learn more about transvection by making use of an observation made a few years ago that a tandem duplication for the bithorax complex, Dp-P5, acts as a powerful transvecting rearrangement. The effect of Dp-P5 on transvection requires the use of a combination in cis, rather than trans, of two other mutants of the bithorax complex, Contrabithorax (Cbx) and Ultrabithorax (Ubx). Briefly, Cbx is a dominant gain-of-function mutant which partially transforms the second thoracic segment (T2) into one resembling T3; for example, wings into halteres. Ubx is a loss-of-function mutant which results in the inverse transformation, T3 towards T2; halteres to wings. That Cbx owes its action largely but not exclusively to an action in cis on Ubx+ (and/or adjoining wild-type alleles of other genes such as bx+ and pbx+) is known from comparisons of Cbx+/Ubx with Cbx Ubx/++, the former having a moderate Cbx phenotype while the latter has only a trace of a Cbx phenotype left (wings being slightly reduced compared to those of wild type). That Cbx has a weak action on Ubx+ in trans was evident since introduction of a gross rearrangement of the transvecting type restored the wings of the cis heterozygote to wild type. In other words, Cbx has a weak effect in trans on Ubx+ but only when the chromosomes are in their normal pairing configuration (Lewis, 1954).

The point of departure in the present work was the finding that Cbx Ubx/Dp-P5 also lacks the trans-acting property of Cbx; in other words this genotype also fails to show even the weak Cbx phenotype found in Cbx Ubx/++. The composition of Dp-P5 can be represented as (++)(++) since it represents a tandem direct duplication of some 10 bands of the region from 89E to 90A including the bithorax complex. That the wild-type wings and T2 of Cbx Ubx/(++)(++) are not due to the extra dose of BX-C genes present in this genotype is now ruled out by finding that adding such an extra dose, not in the form of a tandem duplication but as an insertional duplication in another chromosome, fails to obliterate the weak Cbx phenotype found in Cbx Ubx/++ under normal pairing conditions.

Evidently the pairing of the wild-type BX-C regions within Dp-P5 excludes the pairing of either such region with the opposite chromosome containing Cbx Ubx and we assume that in this way the trans action of Cbx on Ubx+ is abolished. The presumption arises that within a tandem duplication, pairing may be even more intense than between homologous chromosomes. To test this possibility we introduced Cbx Ubx into the more proximal portion of Dp-P5 to give (Cbx Ubx)(+++). When the latter is opposite a deficiency for BX-C, it is found that the Cbx phenotype although still weak is nevertheless significantly stronger than that seen when Cbx Ubx is in one chromosome and the wild-type alleles are in the opposite chromosome. Recently,
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by inducing and utilizing still larger tandem duplications, we have been able to synthesize a (Cbx Ubx)(++) and a (++)(Cbx Ubx) configuration, either one of which when tested opposite a deficiency for BX-C gave the same result as found in the case of Dp-P5. These results suggest therefore that the cooperative effect by which Cbx can activate Ubx+ in trans is even more effective when the two BX-C regions are intimately paired in a tandem duplication than when they are paired but in opposite chromosomes.

References:

215. SUPERABDOMINAL: A NEW DOMINANT GAIN-OF-FUNCTION MUTANT IN THE BITHORAX COMPLEX

Investigators: Shigeru Sakonju, Margit Schardin, E. B. Lewis

Perhaps the most useful of all mutations for studying gene regulation are those which express a cis-dominant gain of function (GOF). In the case of the bithorax gene complex, a plethora of such mutants exists that transform an anterior segment towards a more posterior one. In contrast, the recessive loss-of-function (LOF) mutants of this complex transform a posterior segment towards a more anterior one. GOF mutants which affect either the first abdominal (A1) segment or the second (A2) or the fourth (A4) have already been described (Lewis, 1978).

It would normally be extremely difficult to detect a GOF mutant that would transform A3 towards A4 since these two segments are virtually identical in their patterning of cuticular structures. A deliberate search for such a GOF mutant was carried out against a genetic background in which A3 and A4 can be clearly differentiated from one another in males. The strategy takes advantage of another gain-of-function mutant, Miscadastral pigmentation (Mcp) (or male chauvinistic pigmentation as it is known in local laboratory jargon), found by M. Crosby in this laboratory, who showed that it is a dominant GOF mutant transforming A4 towards A5. As a result, the sexually dimorphic black pigmentation pattern of segments A5 and A6 in males extends forward into A4 but not A3.

Two independent mutants have now been induced with ethyl nitroso urea (ENU) on an Mcp-bearing chromosome; each causes in males a variable and patchy extension of this black pattern into A3 as well as A4. A fine structure analysis of one of these mutants has now been completed. It is a new type of GOF mutant and is given the name Superabdominal (Sab). It lies very close to the right of Mcp and has been separated from it by crossing over. Sab without Mcp turns out not to transform A3 towards A4, but instead, on its own, transforms A3 weakly towards A5 and, at the same time, weakly transforms A4 towards A5 as well. Had Sab been a conversion of A3 towards A4, then removing Mcp would have left both A3 and A4 without black, sexually dimorphic, pigmentation. Therefore, the designation Ultra-abdominal-4 (Uab-4), tentatively adopted in last year’s abstract (Biology 1982, No. 170) for Sab, has been abandoned.

Although Sab shares some properties with Mcp, namely, conversion of A4 towards A5, Mcp carries out this transformation to an extreme degree without affecting A3; Sab, on the other hand, carries out a relatively weak transformation of A4 to A5; yet it is able to transform A3 as well as A4 towards A5. Like Mcp, Sab seems to activate the wild-type allele of infra-abdominal-5, iab-5+, a gene responsible, at least in part, for an A5, as opposed to A4, level of abdominal differentiation. The immediate proximity of Sab to Mcp suggests the possibility of multiple sites controlling the regulation of the iab-5+ gene.

Reference:

216. CEPHALOTERGITIC: AN UNUSUAL MUTANT IN THE BITHORAX GENE COMPLEX

Investigator: Margit Schardin

The mutation Cephalotergite (Cet) was discovered as an X-ray-induced revertant of Contrabithorax (Cbx) by T. Ramey. It is a dominant mutant and results in a variable and partial transformation of the dorsal region of the head into tissue resembling that found in the tergites, or dorsal sclerotized plates of abdominal segments; in
addition, occasionally the ventral region of the eye transforms into tissue resembling the poorly sclerotized lateral and ventral regions of an abdominal segment. Cet is of special interest because its properties suggest that certain genes of the bithorax gene complex become abnormally activated in the head region where the complex is presumably normally totally inactive.

Previous work has shown that Cet is associated with a deficiency extending from 89D to 89E (E. B. Lewis, unpublished) and genetic studies of T. Ramey (personal communication) suggest that the loci of both Cbx and Ultrabithorax (Ubx) are deleted. Recently, confirmation has been forthcoming at the molecular level by W. Bender and F. Karch, who find (personal communication) that in the DNA of the Cet chromosome the deletion extends beyond Ubx to the start of the bxd region. In addition to the Ubx phenotype associated with this deletion, Cet also has a weak Ultra-abdominal (Uab) transformation of the first abdominal (A1) segment towards a more posterior one, presumably the second (A2). The Uab effect is believed to be a dominant gain of function resulting from a position effect of the breakage point in 89E.

Although in extensive crossing-over experiments I have been unable to separate Cet from the associated deficiency, I have been able to recombine Cet with Mcp. On the basis of three crossovers, Cet maps close to the left of Mcp, which lies near the middle of the right half of the complex.

Although their genetic basis has not been clarified, cephalic tergite-like structures have been reported in certain strains of tumorous head (tuh-3) by Postlethwait et al. (1972). Subsequently, Kuhn et al. (1979) described other strains of tuh-3 in which cephalic tergite tissue resembled that of A6 in males and that of A6, A7 or A8 in females. The genetic basis of tumorous head phenotypes is complex owing to a long history of selection of the strains for such effects. Nevertheless, Kuhn et al. (1981) have recently shown that in these strains a recessive mutant effect on male ducts, which may represent a hypothetical infra-abdominal-8 (iaab-8) gene in BX-C, is located in the extreme distal half of the complex; it is presumed that the cephalic tergite effects of tuh-3 lines are also owing to a mutant(s) located somewhere in that part of the complex. Since no sexual dimorphism is seen in the cephalic tergite tissue of Cet flies, evidently such tissue is not of the type found in A5 nor A6. Nor does it seem to involve the A4-type since Cet Mcp/Mcp flies similarly are not sexually dimorphic in the cephalic tergites. There is conflicting evidence that A3 may be the type of cephalic tergite in Cet flies. In the triple mutant genotype, Cet Mcp/Mcp Sab (see Abstract No. 215), although A3 is sexually dimorphic in its pigmentation, the cephalic tergite tissue is not. On the other hand, a recently found mutant, induced with X rays in a Cet Mcp chromosome, not only shows strongly sexually dimorphic pigmentation of A3 (as well as A4, A5 and A6) but it also shows such pigmentation in the tergite tissue of the head. Hence it is possible that Cet involves an A3-type of cephalic tergite; however, an A2-type, or even A1-type, is not ruled out. It is likely that Cet is therefore a different mutant from those described for tuh-3 strains in which the cephalic tissue was of the A6 type.

References:

217. EXCEPTIONALLY LONG-ARMED CHROMOSOMES ARE LETHAL IN DROSOPHILA

Investigator: Loring G. Craymer III

In the course of constructing several structurally novel chromosomes, I noted two cases in which a predicted product could not be recovered. In both cases, the unrecoverable chromosome would have had a major arm length approaching three times normal (wild-type Drosophila melanogaster have five major chromosome arms of approximately equal length). Two series of crosses were carried out to systematically investigate the relationship between length and nonrecoverability. Within each series, chromosome lengths were kept constant from cross to cross and only arm lengths varied. Each cross was
designed to recover a chromosome with a major arm length between two and three times normal, and each cross produced a control class of animals.

The long-armed chromosomes from these crosses fell into three categories: (1) easily recovered, (2) rarely recovered, or (3) not recoverable in adults. Carriers of the rarely recovered chromosome show a variety of phenotypic abnormalities and the chromosomes are poorly recovered in succeeding generations. The abnormalities include rough eyes, scalloped wings, abdominal malformations, partial or complete leg losses, poor coordination, and other symptoms indicative of generalized cell death. The degree of abnormality varied from one long-armed chromosome to the next, and there was a high correlation between the severity of the phenotype and the difficulty in recovering the long-armed chromosome. On close examination, some of the "easily recovered" chromosomes also seemed to cause lowered viability and weakly induced the phenotypic abnormalities.

All of the experimental animals were euploid and the only significant difference from one cross to the next within an experimental series was the structure of the long-armed chromosome. Salivary gland chromosome cytology was consistent with the idea that nonrecoverability was directly related to arm length, but one-fourth to one-third of the mitotic chromosome length is concentrated in the chromocenter of polytene nuclei and accurate length determinations cannot be made in these cells. Total mitotic length can be measured in larval neuroblast figures, however, and crosses were set up to generate larvae for mitotic cytological analyses. The crosses were designed so that only the long-armed chromosomes should be recoverable in third-instar larvae, and larval markers were included in the crosses as an added safety measure to ensure that animals carrying long-armed chromosomes would be unambiguously identified. Several of the easily recovered and several of the rarely recovered chromosomes were analyzed. The results support the idea that increased arm length is responsible for the reduced viability and for the phenotypic abnormalities.

The crosses for the initial cytological analyses were transferred to 18°C instead of the usual 25° in order to delay development so that as many animals could be collected from each cross as possible. A fortuitous result was that it was discovered that the viability and phenotypic effects are somewhat temperature-sensitive. Stocks have been established at 18°C of some of the chromosomes which are not recovered or rarely recovered at 25°C. Further, animals carrying chromosomes which were easily recovered at 25° have decreased viability at 29° and show some degree of the phenotypic abnormalities.

The most attractive hypothesis for explaining the various results is that sister chromosomes overlap or fail to properly disengage during anaphase and either form pseudo-bridges or cause some difficulties when nuclear membranes are formed. At higher temperatures, chemical processes and molecular movements are speeded up so that cell division is a more rapid process and the chromosomes are battered more from bombardment by free molecules. Chromosomes might thus be more easily entangled, and this would lead to the observed temperature sensitivity.

There are some obvious evolutionary consequences of this phenomenon. Past a minimum genome size, multiple chromosomes are necessary for the survival of an organism. The maximum recoverable arm length for any organism is likely to be a function of nuclear and cellular radii, so that the maximum arm length observed in a species is likely to increase slowly with increasing DNA content. Total chromosome number would increase at a faster rate. Within a species, there could be a tendency for chromosomes to have a limited size range since the length problems would encourage uniformity of arm lengths within a species. There should thus be a tendency for chromosome number to increase with increasing genome size. This is not a unique prediction—speciation by polyploidization has the same effect—but there is a concomitant prediction that no (non-holocentric) species with a large genome should have few chromosomes.
Evidence that the midbody segments of BX-C-deficient Drosophila are composed of structures from the first and second thoracic segments

Investigator: Ian W. Duncan

Drosophila that are entirely deficient for the bithorax gene complex (BX-C) die as late embryos in which each segment from the second thoracic (T2) through the seventh abdominal (A7) segment develops with a morphology very close to that of a normal T2 segment. This has led to the idea that T2 is a developmental ground state with respect to the BX-C. That the situation may be more complex, however, is suggested by the results of clonal analysis experiments reported recently by Morata and Kerridge (1981). These authors studied the morphology in the legs of clones of cells homozygous for Ubx, a BX-C mutation which should eliminate all BX-C gene activity in the thoracic region. Morata and Kerridge found that such clones develop as posterior T1 leg when they occur in the posterior portions of the T2 or T3 legs and as anterior T2 leg when they occur in the anterior portions of the T2 or T3 legs. These observations suggest that the midbody segments (T2 through A7) of animals deficient for the BX-C may be hybrid in character, the anterior portion developing as T2 and the posterior portion as T1. A reexamination of BX-C-deficient embryos (Df(3R)P9 homozygotes) has provided evidence in support of this idea. In wild-type embryos T1 has a ventral setal belt of large pigmented denticles at its anterior margin and a patch of much smaller denticles (called the midventral tuft) which lies posterior to the main setal belt. T2 has several rows of very fine denticles at its anteroventral margin and has no midventral tuft. Posterior to the midventral tuft the ventral portions of T1 and T2 appear very similar to one another. In the majority of BX-C-deficient embryos all midbody segments are in their ventral portions indistinguishable from T2. In about 15% of such embryos, however, at least one midbody segment has been found to possess a small patch of midventral-tuft-like denticles. These denticle patches do not appear to belong to the main setal belt of the segment as they are always far posterior to it. The patches are usually small, containing from two to eight denticles, although segments containing up to 37 midventral-tuft-type denticles have been seen. These observations support the idea that the midbody segments of BX-C deficient animals contain both T1 and T2 structures. Why midventral tuft denticles are present only rarely in these segments is not clear, however. Perhaps the intrasegmental boundary between anterior and posterior is not well defined at the embryonic stage and midventral tuft denticles are only occasionally included in the posterior region of T1.


Publications

THE FOLLOWING REPORT IS BY L. ELIZABETH BERTANI, A VISITING ASSOCIATE IN BIOLOGY WHO IS WORKING WITH DR. JUDITH L. CAMPBELL IN THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING.

219. DEVELOPMENT OF A METHOD FOR IMPROVING THE STABILITY OF AMPLIFIED FOREIGN DNA IN BACTERIA

Investigators: L. Elizabeth Bertani, Giuseppe Bertani*, Judith L. Campbell**

Support: The Energy Conversion and Utilization Technology Division of the Department of Energy

The project is concerned with the development of a new method for the transfer of foreign genes to the chromosome of E. coli and for amplification through duplication and polyplication in situ. We expect the stability of the strains thus amplified will be greater than that of standard strains where genetic amplification is through multiple copies of autonomous, cytoplasmic elements.

In preliminary trials to measure the efficiency of integration, we have made use of the plasmid pBR322, a popular cloning vehicle, which does not by itself integrate into the bacterial chromosome. In addition, extrachromosomal replication of pBR322 can be controlled by using a temperature-sensitive polA host, as active polA gene product is necessary for plasmid replication. Hybrid plasmids, consisting of the specific integration system of the temperate phage P2 cloned into the Pst I site on pBR322, were used to transform ts-polA bacteria. When cultivated at 42°C in the absence of antibiotic selection, these bacteria give rise with virtually 100% efficiency to derivatives that, according to the following criteria, could contain a single integrated copy of the plasmid: (1) they are resistant to low (4 µg/ml), but not high (20 µg/ml), levels of tetracycline; (2) a wild-type phage gene, D, also carried on the plasmid, complements an amber D mutant with reduced (0.25) efficiency; (3) the strains are produced only when the hybrid plasmid has an active phage integration system; (4) or they may be produced from a hybrid plasmid that has a defective int system, if int+ gene product is supplied by an infecting phage; (5) the low level resistant strains can be converted to high level again when gene products necessary for phage excision are supplied by an infecting phage.

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**Division of Chemistry, California Institute of Technology.
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.

220. Transcripts of the Six Drosophila Actin Genes Accumulate in a Stage- and Tissue-Specific Manner

Investigators: Beverley J. Bond, Eric A. Fyrberg*, James W. Mahaffey*, Norman Davidson**

Support: National Institutes of Health, USPHS
Muscular Dystrophy Association of America

We have investigated the expression of the six Drosophila actin genes during ontogeny. Unique portions of cloned actin genes were used to monitor levels of respective mRNAs in developmentally staged whole organisms and dissected body parts. We find each gene is transcribed to form functional mRNA, which accumulates with a distinct pattern. Two of the genes, act-5C and act-42A, are expressed in undifferentiated cells and probably encode cytoplasmic actins. Act-57A and 87E are expressed predominantly in larval, pupal, and adult intersegmental muscles; act-60F in muscles of the adult thorax, and act-79B in the thorax and leg muscles. These composite data define three main patterns of actin gene expression which are correlated with changing Drosophila morphology, particularly muscle differentiation and reorganization.

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

221. An Approach Toward Finding Regulatory Sequences in Drosophila Actin Genes

Investigators: Beverley J. Bond, Norman Davidson*

Support: National Institutes of Health, USPHS

Expression of the six Drosophila actin genes is regulated in a stage- and tissue-specific manner (see Abstract No. 220). We wish to determine whether sequences adjacent to a particular gene function as cis-active regulatory elements in controlling differential expression.

For this purpose, we plan to take advantage of the Drosophila P element transformation system devised by Rubin and Spradling (1992). This requires the construction of vectors containing the gene of interest and a selectable marker inserted within the boundaries of the P element. We have chosen the Drosophila alcohol dehydrogenase gene (courtesy of D. Goldberg and J. Posakony) as a selectable marker. The injected actin genes need to be modified in some way to enable their transcripts to be distinguished from the endogenous ones. We have chosen to use "hybrid" actin genes containing the 5' end of one of the genes fused to the 3' end of another. These hybrid genes if expressed should yield transcripts that can be detected on RNA blots with specific probes for the 3' and 5' untranslated regions of the donor genes.

We will initially ask whether the injected hybrid gene is expressed in vivo with a developmental time course similar to that of its 5' parent or its 3' parent. We will then attempt to identify regulatory sequences that play a role in defining their specific pattern of expression.

Reference:
*Division of Chemistry and Chemical Engineering, California Institute of Technology.

222. DNA Replication: The Role of the CDC2 Protein

Investigators: Caren Chang, Judith L. Campbell*

Support: National Institutes of Health, USPHS

We are interested in understanding the enzymology of DNA replication in the simple eukaryote Saccharomyces cerevisiae (baker's yeast). One initial approach to studying DNA replication has been based on genetically-defined mutants. Chia-lam Kuo* screened a population of temperature-sensitive yeast mutants using in vitro replication assay (Kuo and Campbell, 1983). The assay measured incorporation of radioactively labeled nucleoside triphosphates into DNA by Brij-treated (permeabilized) cells. This method identified 19 mutants defective in DNA replication, five of which contained mutations in a previously identified gene called CDC2. CDC2 function has been shown to be required for the completion of S phase by Lee Hartwell at the University of...
Washington. Our assay indicates that CDC2 plays a
direct role in DNA synthesis; however its exact
function has not been established.

We are presently cloning the CDC2 gene by in
vivo complementation with two yeast libraries. One
library is contained on a stable, centromere
plasmid; the other is on an unstable, high copy-
number plasmid. With the isolated gene, we plan to
examine functions such as the expression of CDC2
during the cell cycle and its ability to complement
other replication mutants. Concomitant with this
molecular biology approach, we are purifying the
CDC2 protein. A complementation assay for the
purification is provided
by
an
in
vitro replication
system developed in this lab (Celniker and
Campbell, 1982). Once the protein is purified, the
in vitro system will also be used to elucidate its
function. With the isolation of both the CDC2 gene
and the CDC2 protein, we hope to obtain insight
into a component of replication.

References:
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Kuo, C.-L. and Campbell, J. L. (1983) PNAS, in
press.

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

223. MOLECULAR BIOLOGY OF THE
ACETYLCHOLINE RECEPTOR FROM TORPEDO

Investigators: Katharine S. Mixter, N. Davis
Hershey*, Daniel Noonan*, Toni
Claudio**, Shirley Taylor*,
Norman Davidson*

Support: Muscular Dystrophy Association
of America
National Institutes of Health, USPHS

The electric ray, Torpedo, has been widely used
to study the protein components that make up the
cholinergic synapse because of the highly
specialized electroplaque cells that make up the
electric organ. Studies in the laboratory of
Professor Michael A. Raftery at Caltech as well as
those by other laboratories have conclusively shown
that the acetylcholine receptor is an integral
membrane protein complex composed of four different
polypeptide subunits (Raftery et al., 1980). These
peptides--40 kd, 50 kd, 60 kd, and 65 kd--are found
in a 2:1:1:1 stoichiometric ratio in native
receptors. In order to investigate further the
structure and action of this important class of
neuroreceptors, we have set out to clone the genes
for these four subunits.

We have previously isolated partial cDNA clones
from a cDNA library constructed by standard methods
from Torpedo electric organ poly(A)* RNA. Two such
clones were shown to represent parts of the mRNAs
for the 65 kd and 50 kd subunits by the criterion
of specifically selecting mRNAs for these proteins
from poly(A)* RNA by hybridization.

We have now sequenced these two partial cDNA
clones and shown that they align very nearly with
the published cDNA sequences for these messages
(Noda et al., 1983). Using the published numbering
system, our clones include 1346 nucleotides of the
65 kd subunit's message and 700 nucleotides of the
50 kd subunit's message. These correspond to amino
cacids 118 through the end of the protein coding
sequence for the 65 kd subunit and amino acids 44
through 277 for the 65 kd subunit.

By several subsequent cloning steps using the
above cDNAs as starting material, we are isolating
the complete region of genomic DNA encoding the 65
kd and 50 kd subunits. Genomic clones also are
being isolated for the 40 kd and 60 kd subunits.
The screening of the genomic library is being
carried out by the recombination method of Brian
Seed (unpublished).

The isolation of genomic clones for the four
subunits of the acetylcholine receptor will allow
us to address several interesting questions. We
would like to know whether non-allelic copies exist
for one or more of the subunits. Differences in
the proteins encoded by these multiple genes, if
they exist, could account for some of the differ­
ences between the receptors found on various
tissues.

References:
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Kikyotani, S., Hirose, T., Assi, M.,
Takashina, H., Inayama, S., Miyata, T. and
Raftery, M. A., Hunkapiller, M. W., Strader, C. D.

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York.
Many of the structural genes of higher eukaryotes have intervening sequences. The basic issue we wish to address is whether primary transcripts of eukaryotic structural genes require intervening sequences within them in order to be processed to generate functional mRNAs.

Eukaryotic multigene families provide a good system in which to study the role intervening sequences may play in gene expression. There are six non-allelic actin genes in the haploid genome of Drosophila melanogaster. They share extensive homology in their protein-coding regions, but diverge significantly in both 5' and 3' untranslated regions. They are all expressed, at various stages of Drosophila development. It has been shown that four members of the family have intervening sequences, which occur in three different positions (Fyrberg et al., 1981). The initial goal of this study was to find out whether or not the other two actin genes, denoted Act-42A and Act-87E because of their chromosomal localizations, have intervening sequences. We have performed S1 and ExoVII nuclease mapping of these two genes. Our results indicate that they both contain intervening sequences near their ends, either 5' or 3'. Further investigation to locate these intervening sequences is being carried out by DNA sequencing.

Reference:

*Division of Chemistry and Chemical Engineering, California Institute of Technology.
GRADUATES

FINANCIAL SUPPORT
GRADUATES

Twenty-seven students in Biology were awarded the B.S., M.S. or Ph.D. degree in June 1983. Names, degrees conferred, and titles of doctoral theses are as follows:

Bachelor of Science

Loren Ingrid Alving, B.S. with honor.
Gary Craig Mockli, B.S. with honor.
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Mitchell Kronenberg, Ph.D. Gene Expression in B and T Lymphocytes: I. Evolution of Rat C<sub>κ</sub> Alleles. II. The T-cell Receptor Problem.

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