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**Biology Division Progress Report  
for Period of  
October 1, 1986-September 30, 1988**

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National Technical Information Service  
U.S. Department of Commerce  
5285 Port Royal Road, Springfield, Virginia 22161  
NTIS price codes—Printed Copy:A08 Microfiche A01

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# BIOLOGY DIVISION PROGRESS REPORT

For Period of October 1, 1986 - September 30, 1988

Date Published: September 1988

Fred C. Hartman, Acting Director

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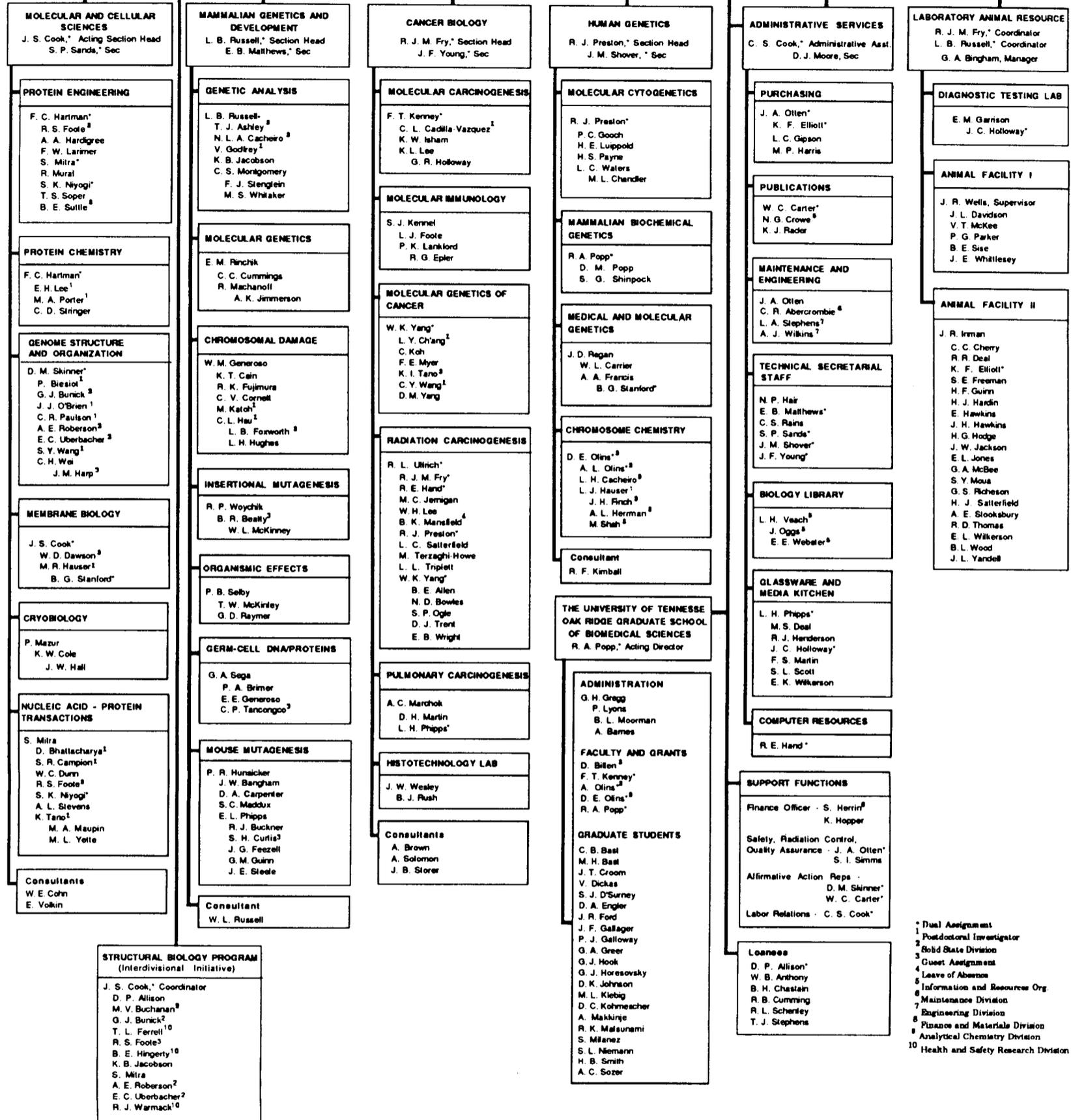
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BIOLOGY DIVISION

July 1988

F. C. Hartman, Acting Director  
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11A

APPROVED: F. C. Hartman

- \* Dual Assignment
- <sup>1</sup> Postdoctoral Investigator
- <sup>2</sup> Solid State Division
- <sup>3</sup> Guest Assignment
- <sup>4</sup> Leave of Absence
- <sup>5</sup> Information and Resources Org
- <sup>6</sup> Maintenance Division
- <sup>7</sup> Engineering Division
- <sup>8</sup> Finance and Materials Division
- <sup>9</sup> Analytical Chemistry Division
- <sup>10</sup> Health and Safety Research Division



## Foreword

The Biology Division of the Oak Ridge National Laboratory is one component of the Department of Energy's intramural program in life sciences. Accordingly, ~75% of the Division's total budget is derived from the Department of Energy through its Office of Health and Environmental Research. With respect to experimental biology, the congressionally mandated mission of this Office is to study adverse health effects of energy production and utilization.

Within this stated broad mission, common themes among the research programs of the Biology Division are interactions of animals, cells, and molecules with their respective environments. Investigations focus on genetic and somatic effects of radiation and chemicals. Goals include identification and quantification of these effects, elucidation of pathways by which the effects are expressed, assessment of risks associated with radiation and chemical exposures, and establishment of strategies for extrapolation of risk data from animals to humans. Concurrent basic studies in genetics, biochemistry, molecular biology, and cell biology illuminate normal life processes as prerequisites to comprehending mutagenic and carcinogenic effects of environmental agents.

Research grants from agencies other than DOE, secured through the initiatives of principal investigators, comprise the remaining portion of the funding base for the Biology Division. Collectively, these grants complement and enhance the DOE-supported activities and provide positions for students, postdoctoral investigators, and research associates, who contribute enormously to the Division's total research efforts.

The premier challenge to the Division for the past decade has been to maintain the diversity and unassailable quality of research programs, despite chronic budgetary erosion with consequential hardships, as required to adapt to evolving needs of sponsors and society. Shifts in emphases that have occurred during the present reporting period are reflected by infusion of molecular biology expertise into the Mammalian Genetics Program, by further integration of molecular biology and biochemistry under the theme of Protein Engineering, and by new initiatives concerning hazards of radon exposure. The Division is striving to posture for increased participation in genome mapping and structural biology, areas targeted for growth by the Department of Energy.

This Progress Report is intended to provide both broad perspectives of the Division's research programs and synopses of recent achievements. Readers are invited to contact individual principal investigators for more detailed information, including reprints of publications.

*Fred C. Hartman*

**Superscripts after Staff Names  
on Research Summaries**

<sup>1</sup>Postdoctoral Investigator

<sup>2</sup>Loan from Solid State Division

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<sup>4</sup>Student, University of Tennessee-Oak Ridge Graduate  
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# Research Activities

## Mammalian Genetics and Development Section

### Section Overview - L. B. Russell

A strong emphasis in molecular genetics has been added to the Section's continuing themes in basic genetics and mammalian germ-cell mutagenesis. E. M. Rinchik, who joined the Section at the beginning of 1986, and R. P. Woychik, who came in October 1987, have established active groups that take different and complementary approaches to the theme of exploring relations between DNA structure and function in a mammalian genome. The evolutionary relationship between mouse and man allows direct extrapolation of many of the mouse findings to humans.

The Molecular Genetics Group exploits existing deletion mutations clustered around loci in different parts of the mouse genome to derive detailed molecular maps for these regions, which jointly encompass 1-2% of the genome. Functional maps for some of the regions already exist from our past complementation studies and are being derived for others. More finely structured functional maps are currently being generated in experiments designed to saturate regions corresponding to large deletions with new point mutations.

The second approach to correlating molecular structure with function is being developed by the Insertional Mutagenesis Group. Transgenic-mouse technology is used to generate mutations by gene disruption. Because the mutant locus is "tagged" with exogenous DNA, it (and, subsequently, its normal counterpart) can be readily cloned and characterized with respect to both structure and expression. Over 50 transgenic founder mice have been generated in the first 6 months of this project, and lines have been derived from many of these.

The value of having preserved mutant mouse stocks, particularly radiation-induced mutations, has become apparent from the molecular studies that utilize deletions and other chromosomal rearrangements to such great advantage. Consequently, the genetic characterization of five specific regions of the mouse genome has been intensified in work that is closely interactive with both in-house and collaborative molecular studies. Complementation analyses, cytogenetic banding of chromosomes, recombination between presumed pseudo-alleles, deletion mapping, and embryological analyses are components of these genetic characterizations.

Another theme with increased emphasis in the Section's research is the exploitation of mouse models for human developmental anomalies or genetic disorders. Detailed clinical, physiologic, immunologic, and other analyses are being conducted in a number of mutant stocks. One exciting study is the analysis of the sex-linked mutant *scurfy* (*sf*), for which a failure in regulation of B-lymphocyte development or function has been

demonstrated. Other studies involve certain unbalanced translocation segregants that were found to provide models for some common human developmental anomalies; several of these mouse models are being exploited in collaborative studies. Dominant skeletal mutations, too, have been found to closely mimic specific human genetic disorders.

Work has continued on the world's largest series of X-autosome translocations, rare types of rearrangements that have arisen in our mutagenesis studies over the past decades. These translocations (in which the X is distributed to two different chromosomes) are being used in studies of X-inactivation, in the molecular mapping of the X chromosome, in the exploration of meiotic-chromosome-pairing mechanisms, in localizing gene expression to certain cell types, and (since they are readily sortable or identifiable for microdissection) as sources of DNA libraries that are enriched for certain regions of the genome.

In the area of mutagenesis, some of the more exciting findings during this reporting period have come from the exposure of zygotes to chemical agents. Exposure near the time of fertilization or shortly afterwards, results in high frequencies of fetal malformations and death, effects that we have been able to show were not mediated by the maternal environment or caused by chromosome breakage or missegregation. Molecular studies in progress are now providing some evidence that the anomalies may come about through changes in gene expression brought about by mutagen-mediated DNA amplification. -- Investigations of the sensitivity of zygotes to the induction of gene mutations by N-ethyl-N-nitrosourea (ENU) have also given exciting results: a mutation rate that is almost an order of magnitude higher than that in spermatogonia, and a very high frequency of mosaics among the mutants. Since somatic/germline mosaics are excellent tools for various basic studies, the ENU exposure of early zygotes may provide a method for producing such biological reagents.

Other types of mutants, too, may now be more readily producible as a result of our recent findings in other mutagenesis experiments. Thus, if high frequencies of intragenic mutations are desired, the treatment of choice is exposure of spermatogonial stem cells to  $4 \times 100$  mg ENU/kg. Moreover, preliminary results indicate that larger lesions (probably including multilocus deletions) can be induced with the highest mutation rate ever recorded in any germ-cell stage by exposing spermatids to chlorambucil. The continuing effort to enlarge the data base for mammalian germline mutagenesis results has revealed vast differences among chemicals with regard to spermatogenic-stage-sensitivity patterns, for both mutagenesis and cytotoxicity. Mutagenesis studies in females have addressed the question of genetic vs maternal causes of embryonic mortality and have identified a chemical that in oocytes produces chromosome breaks primarily in centromeric regions. Another series of experiments has demonstrated that the second meiotic metaphase of the oocyte is a sensitive stage for the detection of agents that induce aneuploidy by spindle interference. New indicators of dominant genetic damage are also being explored, and a recent experiment has combined studies of stunted growth, reduced survival, and skeletal anomalies in  $F_1$ .

The Germ-Cell DNA/Proteins Group has made several important recent findings, the most significant one being that, for certain mutagens, sperm protamine -- rather than DNA -- is the critical target for induction of chromosome aberrations. In addition to its obvious basic importance, this finding also has applications for mutagenicity hazard interpretations; thus, it is no longer safe to conclude that an agent that fails to interact with DNA in other systems will necessarily be innocuous in mammalian germ cells. The group is also exploring alkaline elution techniques for measuring DNA strand breaks in germ cells after exposure to differently acting agents, a method that may eventually prove applicable for monitoring DNA damage in human sperm. Work is continuing on measurement of unscheduled DNA synthesis (UDS) as a sensitive indicator of DNA damage and repair; effects of both rate and route of exposure were recently demonstrated to affect the magnitude of UDS.

While each Group of the Mammalian Genetics and Development Section has its own active program, there are numerous interactions between Groups. A prime example is the close interplay between the genetic characterization of mutations in certain regions of the genome and the molecular analyses of these same regions (Russell and Rinchik groups). Probes developed in insertional-mutagenesis studies are being used to analyze mutations generated in conventional mutagenesis experiments, as in the case of a translocation associated with limb defect (Woychik and Generoso groups). Methods developed for the detection of dominant skeletal mutations are being applied to search for mutations in transgenic lines of mice (Selby and Woychik groups). Patterns of adduct binding to DNA and/or protamines in germ cells are being correlated with patterns of induction of transmissible chromosomal damage (Sega and Generoso groups). Several other examples could be cited. The mutants which are being (and have been) generated in mutagenesis studies are being used as tools by several of the investigators in the Section and in the Division. In addition, 92 groups of mice from mutant and other stocks have been shipped since the time of the last progress report to 20 investigators at other laboratories in the USA and Europe. A large number of collaborative studies with other laboratories have been initiated or continued, and mention of these is scattered throughout the individual reports that follow.

## MOLECULAR GENETICS AND MOUSE GENOME STUDIES

E. M. Rinchik	D. K. Johnson <sup>4</sup>
D. A. Carpenter	M. L. Klebig <sup>4</sup>
C. C. Cummings	R. Machanoff
A. K. Jimmerson	G. D. Raymer

The major interests of the Molecular Genetics Group lie in exploiting spontaneous and induced heritable mutations of the mouse to learn more about the genetic control of normal and abnormal development in mammals. For almost 40 years, germ-cell-mutagenesis experiments at ORNL have generated an impressive collection of various agent-induced mutations many of which are still maintained in breeding stocks. These mutations vary widely in degree of phenotypic complexity, in severity-of-effect, and in structure at the DNA level. An important subset is comprised of radiation-induced lethal mutations, which have occurred at several specific regions within the mouse genome. These types of mutations, which are usually chromosomal deletions of varying lengths, are extremely useful reagents for accomplishing a detailed molecular and functional analysis of approximately 1-2% of the genome. Accordingly, superimposed upon the continuing underlying theme of basic mouse genetics, the Group's efforts fall into three major categories: (1) Molecular mapping and characterization of genomic regions associated with radiation-induced deletion mutations; (2) High-efficiency saturation mutagenesis of these same regions, as a model system for detailed, fine-structure *functional* mapping of large genomic segments; and (3) Molecular genetics of a multiple-allele series at the *Steel (Sl)* locus, as a model for studying complex genetic loci exhibiting mutational dominance and pleiotropy.

### Molecular Genetics of Deletions at Specific Loci

We have continued our initial experiments designed to gain molecular access to complexes of deletion mutations at both the albino (*c*) and pink-eyed deletion (*p*) loci in Chromosome 7. The largest of the 37 *c* deletions is 6-11 cM in length (perhaps 6-20 million bases of DNA), and the largest of the 43 hypothesized *p* deletions is at least 3 cM in length (perhaps >3 million bases). Genetic analysis of the panel of *c*-region deletions has resulted in a functional map that defines genes important for prenatal, neonatal, and juvenile well-being, proper regulation of a host of metabolic enzymes and blood proteins, male and female fertility, and inner-ear development. Analysis of *p*-region deletions is yielding similar information on some of the same sorts of phenotypes.

We have completed the construction of a bank of mapping-panel DNAs for the *c* region derived from animals that carry (1) homozygous or overlapping deletions (for homozygous-viable or complementing combinations of deletions), and (2) homozygous-lethal deletions balanced opposite a *Mus spretus* chromosome. This panel of DNAs will allow the selection of anonymous clones from enriched genomic libraries that map to this region

(by selection of clones that map to the largest lethal deletion), and the rapid submapping of clones, with respect to deletion breakpoints, within the entire panel of deletions. Indeed, we have selected and mapped an anonymous probe from a flow-sorted chromosome library, one probe (23.3) derived from the cloning of an endogenous ecotropic leukemia provirus closely linked to *c*, one probe (in collaboration with Terry Magnuson, Case Western Reserve University) derived from chromosome microdissection and microcloning, and one probe (in collaboration with Jonathan Stoye and Wayne Frankel, Tufts University) derived from the cloning of another *c*-linked endogenous provirus.

These four clones serve as important access points for diverse areas within the 6-11 cM region corresponding to the largest *c* deletion. Some of the clones can discriminate between members of grossly defined complementation groups, and others are being used in large-fragment DNA analysis to identify distances to deletion breakpoints. Importantly, these clones, along with larger stretches of cloned DNAs associated with them, are being placed into intervals of the genetic functional map, which is also currently defined by deletion breakpoints carried in the radiation mutations.

A similar analysis is being carried out for lethal *p*-locus mutations. Currently, 23 lethal deletions (of 43 total) have been balanced opposite *spretus* chromosomes for rapid RFLP mapping of clones. We are also continuing large-fragment DNA analysis of several radiation-induced mutations of the dilute-short ear (*d-se*) deletion complex in Chromosome 9, and have entered into a collaboration with Ian Jackson (MRC Clinical and Population Cytogenetics Unit, Edinburgh) to begin a molecular analysis of the Oak-Ridge series of radiation-induced lethal mutations at the brown (*b*) locus in Chromosome 4.

#### Saturation Mutagenesis of Deletion-Associated Regions

In the past two years, we initiated the pilot of a large-scale mutagenesis experiment designed to "saturate," with presumed point mutations, specific regions of the mouse genome associated with long, radiation-induced deletion mutations (the very regions that are the targets of our molecular-mapping analyses). These experiments are designed (1) to establish an estimate of the minimum number of genes within a region that are mutable to specific, biologically significant phenotypes, and (2) to provide, for several regions of the genome, a *fine-structure functional map*, based on a series of heritable point mutations with characteristic phenotypes, which can subsequently be correlated with a detailed molecular/physical map currently being developed for these same regions; and (3) to provide fundamental genetical and logistical information on which to base strategies for subsequent large-scale expansion of the functional maps of mammalian genomes.

The experiment involves the 6-11 cM region defined by the *Df(c sh-1)<sup>26DVT</sup>* deletion of the *c* locus in Chromosome 7. Males carrying a standard, non-lethal albino (*c*) marker are treated with a highly mutagenic

dose of N-ethyl-N-nitrosourea. Daughters (+/c) are crossed to tester [ $c^{ch} + / Df(c\ sh-1)^{26DVT}$ ] males, and albino progeny  $c / Df(c\ sh-1)^{26DVT}$  are inspected for new phenotypes. Absence of the albino class suggests the presence of a newly induced lethal mutation (on the  $c$ -bearing chromosome) that falls within the limits of the  $Df(c\ sh-1)^{26DVT}$  deletion. Testing of 773 +/c  $F_1$  females has detected two new, confirmed lethals, with four more presumed lethals currently undergoing testing, two repeat mutations of the neurologic locus *shaker 1* ( $sh-1$ ), and one "fitness" mutation, which manifests a runting syndrome. Mapping and complementation analyses with these new mutations are currently under way.

We anticipate that, in addition to refining the functional maps of genomic regions associated with deletions, these new (presumably) individual-gene mutations will also be important for future use as function-deficient (or function-altered) hosts for receiving segments of cloned, wild-type DNA via transgenic-mouse technology. These types of correction-of-phenotype experiments, if technically feasible, will comprise the ultimate strategy of gene identification for expressed DNA sequences derived from these regions.

#### Genetic Analysis of *Steel* (*S1*) Mutations

Specific-locus mutagenesis tests designed to look for induction of mutations at seven recessive tester loci routinely yield a number of *dominant* visible mutations at other loci. The Mammalian Genetics Section has amassed a collection of dominant "light-coat" or "spotting" mutations. One locus of interest that is often associated with dominant mutations of this type is the *Steel* (*S1*) locus in Chromosome 10. Independent mutations at the *S1* locus can have variable effects on hematopoiesis, pigment-cell migration, and gametogenesis. Molecular-genetic analysis of the *S1*-locus region and our large series (>50) of dominant *S1* mutations can address several general and fundamental questions in mammalian developmental genetics. Some of these questions relate to the basic molecular and functional nature of dominant mutations (the type of genetic alteration that is most important, by far, for meaningful genetic-risk estimation); the existence of (and characterization of) "hotspots" for chromosomal translocations; the nature of cell-environment interactions during development; and the nature(s), mechanism(s), and effect(s) of mutational pleiotropy, in which a single mutation influences more than one (apparently unrelated) developmental pathway. Indeed, the *S1* locus, along with the dominant spotting (*W*) locus in Chromosome 5, remains as one of the most useful models of mutational pleiotropy.

The genetic analysis of *S1* mutations is being carried out both by our Group and by the Genetic Analysis Group, (L. B. Russell). The Oak Ridge *S1* mutations vary widely in their effects on each of the developmental pathways described above, and at least five of the mutations are associated with translocations involving Chromosome 10. Continuing genetic experiments are designed to provide some basic information on the genomic structure of a subset of radiation-induced *S1* mutations as a prelude to initiating detailed molecular analyses within this region.

Since many of the Oak Ridge *Sl* mutations arose in radiation-mutagenesis experiments, a substantial number may carry chromosomal deletions that involve the *Sl* locus. Because of the great value of deletion mutations for the genetic and molecular analysis of chromosomal regions and complex genetic loci, we are attempting to identify *Sl*'s that are caused by deletions. One series of these experiments is designed to test whether radiation-induced *Sl* mutations carry other lethal factors, in addition to the lethality caused by "severe" alleles of the *Sl* locus itself. We have now found that homozygotes for at least six prenatally lethal radiation-induced alleles of *Sl* die before the typical "late" (>day 14), macrocytic-anemia-associated lethal period that is characteristic of homozygotes for the original *Sl* allele (i.e., no anemic fetuses can be recovered at day 16.5 of gestation from crosses of heterozygotes carrying these "early-death" alleles).

Detection of these early-death phenotypes suggests that these mutants carry deletions that remove neighboring genes required for normal fetal development to proceed to mid-gestation. The presence of anemic 16.5d fetuses from crosses of two independent early-death *Sl* mutations of this type should then indicate complementation for early death, but not for the basic *Sl*-associated anemia. These individuals might, therefore, carry overlapping deletions immediately surrounding *Sl*, similar to those observed in other genomic regions. Such overlapping deletions could be useful in deletion-mapping of both molecular clones and phenotypes. We have found one early-death mutation that can complement each of the other five mutations for early death (i.e., late, anemia-associated death is observed). However, none of those five mutations will complement each other in any combination.

Direct deletion-mapping experiments with an electrophoretic variant at the closely linked ( $3 \pm 2$  cM) Peptidase-2 (*Pep-2*) locus have demonstrated that five of the six early-death *Sl*'s are not deleted for *Pep-2* (one remains to be assayed). Control crosses, which incorporate the closely linked *Pep-2* marker, will rigorously test whether the *Sl/Sl* genotype for these "early-death" alleles is indeed lethal. This lethality has been assumed, because no obvious *Sl/Sl* phenotypes (distinguishable from phenotypes normally associated with *Sl/+* and *+/+* animals) have been observed in intercrosses of *Sl*<sup>early-death</sup>/*+* animals.

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**MOLECULAR ANALYSIS OF THE MOUSE GENOME UTILIZING PROBES  
DERIVED FROM TRANSGENIC MICE**

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**Insertional Mutagenesis in Transgenic Mice**

Recent advances in gene cloning and embryo manipulation technologies provide avenues to probe the relationship between the basic structure of the genetic material and the physiological, biochemical and behavioral characteristics of the developing and mature organism. Strategies within the Department of Energy to physically map and sequence the entire mammalian genome will unquestionably contribute to this effort. However, nucleotide sequence and physical mapping data alone are totally devoid of the information necessary for locating and determining the size and the temporal/spatial expression of the functional loci within the genome. In contrast, genetic approaches involving the analysis of individual mutations have succeeded historically in establishing the position of genes on the genomes of several organisms, including those of the mouse and man.

A mutant locus must be clonable to be useful to the molecular geneticist. Unfortunately, the dearth of well characterized genomic probes has precluded cloning most of the mutant loci that have been characterized with classical genetic techniques. For this reason, insertional mutations in transgenic mice are becoming increasingly useful as genetic reagents, because the mutant locus is "tagged" with the exogenously added DNA and can thus be readily cloned. The cloned mutant locus then yields probes that are used to clone and characterize the structure and expression of the corresponding wild-type endogenous copy of the gene. With this approach, a molecular correlation can be made between a complex phenotype in the mutant animal and the expression of the corresponding wild-type gene in the normal animal.

Insertional mutations can be readily generated in the mouse using the pronuclear microinjection transgenic technology. With such an approach, cloned DNA fragments, which have been microinjected directly into the male pronucleus of the fertilized egg, will integrate into the host DNA to

become a stable heritable part of the genome. Multiple copies of the microinjected DNA fragment most often integrate at a single site within a given transgenic mouse, usually in a head-to-tail arrangement. Site selection for integration appears to be entirely random throughout the host genome. Based on current estimates, in 10-20% of the individual lines of transgenic mice, integration of the exogenously added DNA will interfere with the expression of a gene within the host genome and will give rise to a detectable phenotype in the whole animal. Most mutations generated in this manner are recessive and give rise to phenotypes ranging from embryonic lethalties to limb deformities. The mutant locus can be cloned by utilizing the exogenously added DNA fragment as a molecular "tag."

Our unique animal facilities have permitted us to launch a large-scale insertional mutagenesis program. Annually, we expect to produce 200 different lines of transgenic mice, which will be screened in a systematic manner for a variety of phenotypes ranging from embryonic lethalties to skeletal abnormalities. The DNA fragment that is being microinjected to generate these lines of mice contains the reporter gene, chloramphenicol acetyl transferase (CAT). Expression of the CAT gene in the various tissues of the animals will be assayed with a simple and highly sensitive biochemical procedure which can quickly establish whether an individual animal contains the transgene (by performing a CAT assay on tissue derived from the distal portion of the tail). Additionally, the insertion site on each line of mice will be mapped and used as a clonable genetic marker for a specific position of the mammalian genome. Many of these markers should be useful as starting points to "walk" to a number of the radiation- and chemical-induced mutations that exist within the stocks of animals being maintained in our section. Cloning the flanking sequences from those transgenic lines that express a specific phenotype will be facilitated by the antibiotic resistance activity of the CAT gene product in *E. coli*.

During the first six months of this project, we have assembled both a state-of-the-art embryo-injection facility and a fully-equipped molecular biology laboratory. Our microinjection procedures have been highly successful, and we have already produced over fifty different lines of transgenic mice. During the next several years, our efforts should produce a large number of clonable mutations and/or well characterized molecular markers scattered throughout the mammalian genome. The close evolutionary relationship between the human and mouse will allow us to use our stocks of mutant mice as animal models for indirectly studying both developmental phenomena and genetic diseases in humans. Our experiments will complement the physical mapping and sequencing activities associated with the Department of Energy's Genome Initiative and will significantly contribute to the establishment of a functional map of the human genome.

## Molecular Analysis of Structural Rearrangements in the Mouse Genome Using Probes Derived from Transgenic Mice

Mutations that have been generated by the Mammalian Genetics Section and propagated as stocks give rise to a broad range of phenotypes. Many of them have been mapped to specific regions of the genome. We are interested in conducting molecular analyses of some of these mutations in order to determine the structure of the normal genes that are associated with the observed phenotypes and to characterize the changes in DNA structure in the mutant genes. In conjunction with our insertional mutagenesis program in transgenic mice, we are developing a number of well-characterized probes that each map to a specific region of the mouse genome. We are finding that these probes can also be useful for analyzing non-transgenic mutations within the same locus and for "walking" to other mutant loci that map close to the probe.

Experiments currently under way in our laboratory illustrate the utility of using probes derived from transgenic mice for the molecular analysis of induced mutations. One such mutation contains a complex rearrangement involving Chromosomes 2 and 17. The exact karyotypic nature of this rearrangement is still under investigation. In the homozygous condition, this mutation exhibits two distinct phenotypes that appear to be allelic to the nonagouti (*a*) and limb deformity (*ld*) mutations, which are about 20 cM apart on Chromosome 2. Using probes that we derived from the *ld* insertional transgenic mutation, we have been able to establish the position of a DNA alteration in one of the major transcriptional units at the *ld* locus of the mutant. This change in the DNA causes a loss of expression of some of the alternately processed forms of the mRNA that are capable of being expressed from this gene. In addition to furnishing information about the mutant, this result provides independent evidence that the gene associated with the insertional mutation within the transgenic line of mice is likely to be responsible for the observed phenotype. We are continuing to use the *ld* probes from the transgenic mouse to characterize the overall structure of the rearranged locus in the mutant. Ultimately, we should have more information concerning the relationship between specific genes in this region of Chromosome 2 and the development of the observed phenotypes in the mutant animals.

## GENETIC ANALYSIS

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### Genetic and Functional Analyses of Chromosomal Regions Surrounding Specific Loci: Interactions with Molecular Studies

The great value of genetically characterized deletions for molecular studies on DNA structure -- and eventually for structure-function relations -- became apparent from our analyses of mutations in the *d-se* (dilute-shortear) region summarized in the last progress report. With our new program in Molecular Genetics now in place (see elsewhere, this report), our efforts in genetic analyses of mutations at specific loci have been intensified. The value of having preserved mutant stocks generated over decades has become amply apparent, especially in the case of radiation-induced mutations, an appreciable proportion of which we now know to be deletions. In work that is closely interactive with molecular studies, we have recently made progress in genetically characterizing several specific regions of the mouse genome.

(1) **Pink-eye (*p*) region, Chromosome 7.** An intercross matrix for 43 radiation-induced *p*-locus mutations (42 of them pre- or postnatally lethal) is being compiled for various parameters, and a preliminary complementation map has been constructed. The results indicate that there are at least 16 complementation groups and probably 6 prenatal-lethal and 2 postnatal-lethal functional units ("loci") surrounding the *p* locus. Several of the mutations do not fit a linear map of complexly overlapping deletions and may represent other small rearrangements. One of our *p*-locus mutations, which we have shown to be deleted for neighboring markers *ru-2* and *Ldh-1* (as well as for *p* and for more than one "lethal" locus), was shown by us to lack the G-dark band 7C and part of the adjacent G-light band 7B5 in mitotic chromosome preparations, thus localizing the region cytogenetically. The *Ldh-1* gene was cloned subsequent to our demonstration that of many mouse genomic DNA fragments that hybridize with a partial LDH cDNA, one maps consistently within the *ru-2 Ldh-1 p* deletion. *Saa* (mouse serum amyloid gene complex) and 24.2 (an anonymous sequence from human Chromosome 11p) also mapped within this long deletion.

(2) **Agouti (*A*) region, Chromosome 2.** We had earlier shown that one of our radiation-induced *A*-locus lethal mutations, *a<sup>x</sup>*, recombines with *A<sup>Y</sup>* (lethal yellow) -- formerly considered to be an allele of *A* -- the recombinant being agouti that is homozygous viable. Further analyses of several of our stocks has now revealed recombination between *A<sup>Y</sup>* and *a<sup>t</sup>* (black-and-tan), and between *A<sup>Y</sup>* and *a* (non-agouti). In each case, the recombination frequency is of the order of 0.1%. We have proposed

that  $a^x$ ,  $a^t$ ,  $a$ , and  $A$  may be true alleles, and that  $A^Y$  may be a pseudoallele that is located 0.1 cM proximally and exerts a *cis* position effect on  $A$  (and possibly on the other  $A$  alleles), thus producing both the yellow phenotype (and associated physiological properties) in heterozygotes and the early embryonic lethality of homozygotes. Copeland, Jenkins, and co-workers (NCI-Frederick Cancer Research Facility) found an ecotropic murine leukemia provirus, *Emv-15*, to be closely associated with  $A^Y$  (but not causative of the  $A^Y$  phenotype), and Siracusa in their lab (with whom we are collaborating) is utilizing this provirus to gain molecular access to the region. Mapping studies of various types show that *Emv-15* lies within 0.3 cM of  $A^Y$ . Our interpretation of the data indicate a location either proximal to  $A^Y$  or distal to  $A$  ( $a$ ,  $a^t$ ,  $a^x$ ), but not between the two. We have initiated a complementation analysis of  $A$ -locus lethal mutations and have found that some make lethal combinations with  $A^Y$  and others with  $a^x$ . None of the deletions tested to date include the closely-linked marker *bp* (brachypody;  $a - bp = 0.3cM$ ).

(3) **Dilute-shortear (*d-se*) region, Chromosome 9.** In earlier work, our extensive genetic mapping of this region facilitated molecular analysis (with entry via the integrated provirus *Emv-3*) that found the structural (molecular) map to be concordant with the functional (complementation) map. With molecular studies continuing in the laboratory of Jenkins and Copeland as well as our own, we are further refining and enlarging the genetic map. We have carried out a large number of crosses with *ash* (ashen), which maps 1 cM proximal to  $d$ , and found about one-third of the *Df(d se)* mutations but only one  $d^{p1}$  mutation to be also deleted for *ash*. Having extended the analyzable region in a proximal direction, we are testing *Df(ash d)* and *Df(ash d se)* mutations with *Pk-3* (pyruvate kinase-3), using an electrophoretic variant. To date, results have been negative. One *Df(ash d se)* and one *Df(d se)* mutation were analyzed in high-resolution Giemsa-banded preparations: both lack parts of bands 9D and 9E1, thus localizing the *d-se* region cytogenetically.

The only known suppressor mutation in a mammal, *dsu* (dilute suppressor, Chromosome 1), is being tested by us in a number of combinations of  $d$ -locus mutations. We find that noncomplementing combinations are unaffected by *dsu/dsu*, i.e., *dsu* does not suppress prenatal lethality. Combinations that, in the absence of *dsu/dsu*, produce a dilute-opisthotonic phenotype (i.e., complement for prenatal but not postnatal lethality) become non-dilute opisthotonic, i.e., *dsu* suppresses the pigment phenotype but not the neurological defect or the postnatal lethality. In view of contrary findings with suppressors in other organisms, it is of interest that *dsu/dsu* can suppress a phenotype associated with a homozygous deletion (brought about by overlapping deletions), supporting the view that  $+^{dsu}$  may furnish a gene product similar to that associated with  $+^d$ .

(4) **Albino (*c*) region, Chromosome 7.** Our complementation map has been further refined in collaborative studies with Terry Magnuson's laboratory (Case Western Reserve University). Embryological analyses

indicate that one of the functional units for implantation survival can be divided into two functions -- formation of extraembryonic ectoderm and formation of embryonic ectoderm. The simplest interpretation of the map indicates that sequences associated with the former are located distally to sequences associated with the latter. On the basis of these results, the number of complementation groups can be expanded from 13 to 14.

(5) *Steel (Sl) region, Chromosome 10.* The *Sl* locus is of major interest in that it controls developmental pathways concerned with erythropoiesis, pigmentation, and germ-cell formation. As part of a long-term study, we have proceeded with our genetic analyses of over 60 presumed *Sl* mutations that have arisen in our laboratory during the past 30 years, about 10% of them spontaneously and the remainder in the progenies of mutagenized mice. Some of the presumed *Sl* mutants have turned out to involve mutations at *W* or another locus instead of *Sl*; however, over 50 are now proven *Sl* alleles. At least 5, and possibly 6, of the latter group (including one of spontaneous origin) are apparently inseparably associated with reciprocal translocations that have one breakpoint in, or very close to, *Sl*, the second chromosome involved being different in each of these translocations. High-resolution G banding in one of these translocations shows the Chromosome-10 (presumed *Sl*) breakpoint to lie in 10D1. A collaborative molecular study of these translocation Steels is under way with Lisa Stubbs in Hans Lehrach's laboratory (Imperial Cancer Research Fund Laboratories, London). The remaining *Sl*-locus mutations encompass a variety of classes: fully viable, partly viable, neonatally lethal, and prenatally lethal. The last class is of particular interest, because it might include deletions, and is being studied further by our Molecular Genetics Group (see abstract elsewhere in this report). The homozygous viable *Sl* mutations have little or no hair pigment but fully pigmented eyes. In the postnatally subviable and neonatally-lethal mutants, anemia is evident. Several patterns of fertility disturbance are represented among the different viable *Sl* mutant stocks.

#### The Use of X-Autosome Translocations as Tools in Various Studies

Among the large numbers of chromosome rearrangements that have been recovered by us over several decades of mutagenesis studies with various agents is the world's most extensive series of X-autosome translocation [T(X;A)s] -- rare types of rearrangement -- which played a major role in our development of the single-active-X-chromosome hypothesis. We have continued to use these as tools in studies of gene inactivation, molecular mapping of the X chromosome, chromosome pairing mechanisms, gene expression, and for other purposes.

T(X;A)s provide excellent tools for studying the location of the X-inactivation center(s) or region(s), because the rearrangement has distributed the X to two different chromosomes, the  $X^A$  and the  $A^X$ , and because regions of the As, as well as of the X, can be inactivated. The question can be asked whether, in those cells in which the intact X is active, both  $X^A$  and  $A^X$  are inactive or only one. On the basis of evidence

from various autosomal markers, we had in the past concluded that an inactivation center or region was located in the central portion of the X, between the R2 and R6 X-chromosome breakpoints [R2 and R6 designating different T(X;A)s]. More recently (see last Progress Report), we measured the activity of OTC (ornithine transcarbamoylase) which is coded for by the *spf* (sparse-fur) gene located close to the centromere of the acrocentric X. Two T(X;7)s and two T(X;4)s were studied; one member of each pair has a long  $X^A$  (containing the R2-R6 region) and a short  $A^X$ , while the other has a short  $X^A$  (lacking the R2-R6 region) and a long  $A^X$ . In both the T(X;7) and T(X;4) of the former type, OTC activity was subject to inactivation, as expected. In the T(X;7) of the latter type, there was full OTC activity, confirming our earlier conclusion (based on autosomal markers) that the R2-R6 region contains the inactivation center(s). However, in the T(X;4) with the short  $X^A$ , OTC was subject to inactivation, which would indicate the presence of more than one inactivation center (since the  $A^X$  is also known to be subject to inactivation). Because of the possibility that the X-chromosome breakpoint mapping of this T(X;4) might have been in error, we have carried out an *in situ* hybridization study with a mouse OTC probe (performed by UT-ORNL graduate student Dabney Johnson). Preliminary results indicate that *spf* is indeed located in the short  $X^A$  and that the original breakpoint mapping was correct. Because of the potential importance of this finding, the OTC activity measurements will be repeated.

Four of our T(X;A)s were used in a collaborative study with Philip Avner (Pasteur Institute, Paris) to map the X chromosome by molecular techniques. Mouse-hamster somatic cell hybrid lines were constructed from T(X;A) males (no intact X present), and one translocation chromosome was retained by selection for  $Hprt^+$  (while most other mouse chromosomes are lost from the cell hybrids). The cell lines were analyzed enzymatically or by Southern blots for absence or presence of various X-chromosome markers or probes in the retained translocation chromosome. Results of the study have localized X breakpoints for a T(X;12) and a T(X;2), neither of which we had previously mapped genetically, and have more finely mapped the breakpoints for one of our T(X;4)s and one of our T(X;7)s, as well as for T(X;16)16H. The hybrid cell lines will allow future rapid mapping of DNA probes relative to the various X-chromosome breakpoints.

The T(X;A)s can provide tools for gaining an understanding of the regulation of chromosome recognition and synapsis. In collaboration with Terry Ashley (University of Tennessee), we have continued synaptonemal-complex analysis of a series of T(X;A)s, and have compared the synaptic (meiotic) behavior of these translocations with their G- and C-band (mitotic) cytogenetics. Ashley has proposed that those rearrangements that have both breakpoints in G-light bands exhibit only homologous synapsis and normal crossing over, while those that have one or both breakpoints in or at the border of a G-dark band exhibit nonhomologous synapsis and suppression of crossing over. Indeed, a recent careful study of four T(X-7)s with different breakpoints revealed a close correlation between the amount and direction of nonhomologous synapsis and

the width and position of the pertinent G-dark band. Another synaptonemal-complex study on two T(X;4)s showed that bands depicted as "stippled" on the standard G-band map behave like G-dark bands with regard to breakpoint location.

In collaboration with a group at the NCI-Frederick Cancer Research Facility, we have used T(X;A)s to study the expression of proto-oncogenes. In adult mice, *Mos* is expressed almost exclusively in the gonads. T(X;A)s block spermatogenesis beyond pachytene or diplotene of meiosis, but, in contrast with other mutants in which spermatogenesis does not proceed past diplotene (*hpg*, *Tfm*), they do not affect premeiotic germ cells. They thus served to provide convincing evidence that *Mos* transcripts are localized to spermatids. The pattern of expression of the testis-specific transcripts for three other genes (*Abl*, actin, and *Hox-1.4*) was found to be similar to that for *Mos*.

T(X;A)s have provided important tools for our Molecular Genetics Group (see their abstract) which is striving to derive Chromosome-7-region-enriched libraries of random clones to be mapped to large deletions. The fact that the X<sup>7</sup> chromosome in two of our T(X;7)s is 1.4 times the length of the longest normal chromosome facilitates fluorescence-activated chromosome sorting of the X<sup>7</sup>, as well as microdissection and microcloning of the pertinent regions of this chromosome.

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#### ENZYME REGULATION IN MOUSE MUTANTS

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Diverse investigations are described because of altered directions of my research during the past two years. A program being eliminated is the biochemical genetics in *Drosophila*. The *Drosophila* studies involved suppression of *v*, the locus for tryptophan oxygenase, and of *pr*, the locus for an enzyme in the pteridine biosynthetic pathway; publications listed below of suppression of *v*, a new assay for tryptophan oxygenase, the overall aspects of *Drosophila* suppression, and eukaryotic suppression were related to this study.

As a biproduct of studies on pteridine biosynthesis, a new class of pteridines was discovered and characterized, the affinity of many biologically active pteridines to C-18 type cartridges was ascertained, and an improved method to prepare *E. coli* GTP-cyclohydrolase was reported. This enzyme is used to prepare the substrate for several enzymes that are involved in pteridine biosynthesis. A study that involved GTP cyclohydrolase from mouse, rat, and *Drosophila* was undertaken to determine whether this enzyme from each organism was subject to feedback regulation by sepiapterin and 6-acetyldihydrohomopterin or to other types of regulation.

New areas of study are being established in biochemical genetics of the mouse. These include a study of the mechanism of X-chromosome

inactivation by employing X-autosomal translocations induced by mutagens. The *sparse-fur* locus on the X chromosome is the structural gene for ornithine transcarbamoylase, and the behavior of fragments of the X chromosome were monitored by measuring the activity of this liver enzyme. Another study in mice was to employ the enzyme pyruvate kinase to measure the extent of deletions that have occurred on Chromosome 9. Two other mouse projects relate to known or possible abnormalities in pteridine biosynthesis and function. The dilute-opisthotonic mutant is being studied by measuring the rate of clearance of phenylalanine from the serum. This mutant is very slow to perform this clearance; a determination of the enzymatic basis for this deficiency is desirable, because phenylketonuria can have several different enzyme deficiencies, any of which cause mental retardation in humans. The second pteridine project is a study of the *hph-1* mutant. We have shown that the deficiency for GTP cyclohydrolase activity is more severe than had been reported, 2% of normal vs the 10% reported.

An entirely different way of probing the genome is by use of the scanning tunneling microscope; support from laboratory overhead has been obtained to explore the possibilities in collaboration with T. Ferrell (Health & Safety Research Division), who has established a STM laboratory at ORNL. This technique has many uncertainties with respect to biological application, so tobacco mosaic virus is being examined as a model system. Initial results are promising.

Toxic metals are one topic under study in collaboration with J. E. Turner and M. W. England (HSRD), and we are examining the genetic and biochemical basis for differences between strains of *Drosophila* that are resistant or sensitive to cadmium. In another collaboration with J. E. Turner and H. Yoshida (HSRD), the theoretical and chemical mechanisms of radiation damage to peptides and nucleic acids are in progress.

As an outgrowth of the toxic metal study, a technique for analysis of metallothionein in fish liver has been developed in collaboration with J. McCarthy and L. Shugart of the Environmental Sciences Division.

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#### CHARACTERIZATION OF MUTANT MOUSE STOCKS

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The Mammalian Genetics Section possesses a rich collection of mutant mouse stocks. Some derive from spontaneous mutations recognized in existing stocks while others were generated by experiments with irradiation and chemical mutagens. These animals provide a valuable resource for the investigation of basic biologic processes and especially for the pathways between altered genetic structure and altered phenotype. They can also furnish animal models for human disease. We have begun characterization of several mutant stocks by evaluation of clinical observations (lifespan, growth, fertility), gross and microscopic morphology, hematology, clinical chemistry, primary cell cultures, and *in vitro* immunologic assays.

The *scurfy* mutation has a sex-linked recessive inheritance and maps to the extreme proximal portion of the acrocentric X chromosome. Clinical characteristics include scaliness and crusting of the eyelids, ears, and extremities, anemia, cachexia, and rapid death (ave. 24 d). The typical morphologic lesion is a lymphohistiocytic proliferation and infiltration of the lymph nodes, spleen, liver, and dermis. Many cells in the cutaneous lesions contain immunoglobulin as identified by immunohistochemical staining. There is a marked polyclonal gammopathy and Coombs-positive hemolytic anemia. The lesions in the *scurfy* mouse are suggestive of a failure in regulation of B-cell development or function.

Other projects in progress include morphologic evaluation of the mutants limb deformity (of which at least four separate occurrences are known, including one originating from insertional mutagenesis and one associated with a translocation), of a new allele of shaker-1 found in a saturation-mutagenesis experiment by E. M. Rinchik, and of juvenile lethal dwarfism, a phenotype associated with deletions involving a functional unit near the *c* locus.

#### CHROMOSOMAL DAMAGE

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#### High Frequencies of Fetal Anomalies Produced Subsequent to Mutagen Treatment of Zygotes - A New Phenomenon in Mammalian Mutagenesis

There are three general methods for experimental induction of developmental anomalies in mammals. First, high frequencies of congenital anomalies are generated by exposing the embryo *in utero* to teratogens. The type of anomaly produced is closely related to the stage of exposure, and the highest overall yield of anomalies occurs when exposures are given during the period of major organogenesis, between days 6 and 13 of gestation in mice. Second, certain mutations that arise following pre-mating exposure of parents to mutagens are expressed as congenital anomalies. This class of induced congenital anomalies has been observed only at low frequencies. And third, integration of transgenes (exogenous DNA sequences) into the mouse genome has proved to be a valuable method of producing developmental mutants for basic studies.

We have discovered yet another effective method of inducing congenital anomalies. High frequencies of fetal malformations and death were induced when female mice were exposed to the mutagens ethylene oxide (by inhalation) or ethyl methanesulfonate (by i.p. injection) near the time of fertilization of their eggs or during early stages of zygote development. The effects were absent or minimal when females were exposed either before copulation or after the fertilized eggs have progressed to the stage of pre-cleavage DNA synthesis or beyond. In studying the possible mechanisms for this phenomenon, we performed a reciprocal zygote transfer experiment, which showed that the effects were not mediated by the maternal environment, and a first-cleavage cytogenetic study, which showed the absence of obvious numerical nor structural chromosome anomalies. Thus, while the evidence suggests a genetic basis for the

fetal anomalies, the nature of the damage is of a type that has not yet been identified in mammalian mutagenesis.

The zygote-derived developmental anomalies could be the result of changes in gene expression. To investigate this possibility, we are attempting to determine whether the mutagen might have brought about insertion of endogenous transposable elements into new sites in the genome, thus affecting the regulation of genes around the insertion sites (i.e., a mechanism analogous to insertion of exogenous DNA sequences in transgenic mice). Following exposure of zygotes to ethyl methanesulfonate, we are analyzing genomic, EcoRI-digested DNAs from individual 12-day (and older) embryos by hybridizing them with the retrotransposon probe MRL (MboI repetitive long terminal repeat) and looking for RFLPs (restriction fragment length polymorphisms) (MRL was cloned by W. Yang in our Division). We are also investigating the possibility that a presumably male-specific band (14 kbp) is normally amplified at a certain stage of fetal development and that the temporal programming of this amplification might be altered by mutagenic treatment of the zygotes

The fetal malformations produced in these studies are generally similar to common human sporadic defects, for which the etiology is usually unknown. Thus, the new phenomenon is of major interest both in chemical safety evaluation and in studying the etiology of congenital abnormalities. Not only does it raise questions regarding the vulnerability of human zygotes when the mother is exposed to environmental chemicals shortly after conception, but it may provide a model for the class of congenital malformations in humans for which the etiology is still unknown.

#### **Mouse Models for Understanding Human Developmental Anomalies**

Mutagenesis research in mice has two major objectives: genetic risk evaluation and understanding basic mammalian biology. The ultimate goal is to apply the findings to human health problems. In both mice and man, mutations often cause developmental anomalies. Understanding the series of processes that lead any given mutation to express a congenital anomaly is one of the most difficult challenges in mammalian biology. Studies to elucidate these complex processes are generally not feasible in humans. Analogous mutants in laboratory mammals provide the best opportunity to probe into the basic biology of developmental defects. Chromosome rearrangements constitute the major class of transmitted genetic damage produced in mice by many environmental mutagens. Many of these rearrangements generated in mutagenesis research in our laboratory have been maintained as stocks, which provide valuable material for the study of developmental anomalies.

Most individuals heterozygous for a balanced reciprocal translocation do not, themselves, exhibit an abnormal phenotype other than reduced fertility, which results either from impaired gametogenesis or from the production of gametes with unbalanced chromosome complements that

lead to early embryonic death. The embryonic death caused by most cases of chromosomal unbalance is so early as to have little or no medical importance. Certain unbalanced segregants, however, live longer and exhibit congenital malformations. Obviously, this class of segregants constitutes a medical burden in humans. A systematic study in our laboratory reveals that translocations that produce this class of segregants are far more frequent than previously realized. Consequently, we are engaged in determining the cytogenetic nature of such rearrangements and the meiotic processes that are responsible for the appearance of this class of unbalanced segregants. In addition to being the subjects of fundamental studies, these translocations are a dependable source of specific malformations that can be useful as models of human disorders and can provide material for morphogenetic studies that cannot be done in human subjects. Two of the several translocation stocks in which certain ones of the unbalanced segregants exhibit serious malformations are described below.

In one of these stocks, one class of unbalanced segregants exhibits clefting of the palate which extends externally along the snout. Facial clefting of this type also occurs in humans. We are collaborating with Kathy Sulik of the University of North Carolina Medical School at Chapel Hill in a study of the morphogenesis of this defect, and we are comparing our rearrangement stock with a transgenic stock of mice that has similar facial clefting. The other translocation stock produces two distinctly different classes of abnormal segregants. One exhibits cranial tube defect (exencephaly); the other exhibits small size and cleft palate. The pathogenesis of these defects, which are common in humans, is not known. In collaboration with Joe C. Rutledge of the University of Texas Health Sciences Center in Dallas, we are studying whether exencephaly arises directly from faulty bone structure in the skull or indirectly from a defective neural tube.

For both translocation stocks, we have determined the meiotic processes and the chromosomes that are responsible for the defects. The exencephalic and facial-clefting segregants appear to have resulted from adjacent-1 segregation, the important imbalances being the small terminal deletions. The small fetus with cleft palate, on the other hand, appears to be the result of alternate segregation with the inclusion of a very small exchange product that does not usually join in multivalent association.

In addition to translocations that produce unbalanced segregants of a type characterized by congenital anomalies, we are also studying certain translocations that in the balanced state are associated with either dominant or recessive effects. These stocks are also used in the molecular studies of R. P. Woychik.

There is currently an upsurge of interest in mouse mutants associated with developmental defects for studies of abnormal and normal mammalian development, underlying genomic structure, and homologies with man. Chromosomal rearrangements are particularly valuable because the

breakpoints mark the genetic location of the defect and provide a convenient starting point for molecular studies. Our systematic collaborative approach combining genetic, cytogenetic, anatomical, and molecular studies on certain stocks such as those described above, should contribute to the understanding of the complex pathway involved in abnormal development.

#### **Mechanisms for Chemical Induction of Heritable Chromosomal Aberrations**

Transmitted numerical and structural chromosome anomalies are the predominant cause of human developmental and birth defects. The major objectives of this program are to understand the mechanisms by which mutagenic chemicals induce these types of genetic changes in mice and to determine how various factors, such as sex, germ-cell stages, and reaction properties of chemicals affect mutagenesis qualitatively and quantitatively.

**Unique mutagenicity of acrylamide.** The overall results from *in vitro* studies of various laboratories indicated that the vinyl monomer, acrylamide, is not a mutagen. Our recent results in mice, on the other hand, proved that it is effective in inducing dominant lethals and heritable translocations in late spermatid and early spermatozoa. While DNA is undoubtedly the generally accepted target molecule for chromosome-breakage effects, G. A. Sega has hypothesized that, for certain chemicals, protamine might be the critical target for the induction of dominant lethals and heritable translocations. This hypothesis was strengthened by his finding that acrylamide does not bind significantly to the DNA of the sensitive germ-cell stages, but does bind well to the protamines (see elsewhere, this report). We now have dominant-lethal data and partial heritable-translocation results for two dimers of acrylamide (methylene and ethylene bisacrylamide), which show that the spectra of sensitive germ-cell stages do not parallel protamine synthesis as in the case of acrylamide monomer. Molecular dosimetry studies with these dimers should reveal the relation between binding to macromolecules and genetic response.

**Mutagenic response in females but not in males.** For a number of chemicals, we have observed induction of presumed dominant lethals in females but not in males. Since the entire organism, and not just the germ cells, was exposed to the chemicals, the question arises as to whether the increased embryonic mortality was due to genetic effect of the chemical in the oocyte or was caused indirectly through maternal toxicity. If the cause was genetic, what is the basis for the sex difference? We have made significant progress on both questions using the compound platinol (cisplatin). Platinol increased early embryonic mortality when females were treated prior to mating (treatment of maturing oocytes). A reciprocal egg transfer experiment ruled out maternal toxicity as the cause, and cytogenetic analysis of first-cleavage metaphases revealed a high incidence of chromosomal deletions and rearrangements. The results of both of these experiments thus provide evidence that the early embryonic mortality results from genetic effects

induced in oocytes. Most interestingly, the breaks and rearrangements involve primarily the distal ends of the chromosomes. Although chromosome banding has yet to be done, it does appear that the centromeric regions are the primary target of platinol. It is possible that this target specificity may be associated with the state of condensation of the maturing oocyte chromosomes, and this may have a bearing on the differential response of the sexes.

**Progress in aneuploidy research.** While practical and sensitive methods exist in mice for detecting induced heritable gene mutations and clastogenic damage and for studying the mechanisms involved in germ-cell mutagenesis, similar methods for heritable aneuploidy have not been available. However, we have made progress in developing methods that will detect the results of germ-cell nondisjunction in developing embryos and in offspring that have survived to stages at which the damage would cause human suffering.

At the time of ovulation, the mouse egg is at the metaphase-II stage of meiosis. It remains in this stage until triggered by sperm entry to complete meiosis. Chemicals that affect the formation and function of organelles involved in chromosome segregation are potential aneuploidy inducers in this germ-cell stage. We speculated that if the metaphase-II spindle of the newly ovulated oocyte is interfered with before sperm entry, this may lead to chromosome nondisjunction in the female genome. The idea was tested with nocodazole, a compound that interferes with the assembly of the spindle microtubules by inhibiting the polymerization of tubulin. When normally ovulating females were treated shortly before fertilization of their eggs, a majority of the resulting embryos died at around the time of implantation. Subsequent cytogenetic analysis of first-cleavage metaphases following nocodazole treatment of metaphase-II oocytes revealed a high frequency with abnormal chromosome numbers, including changes in ploidy. Treatment of females after their eggs had already completed meiosis had no effect whatsoever.

The *in vivo* metaphase-II oocyte nondisjunction method promises to be useful in screening for aneuploidy-inducing chemicals. More important, it paves the way toward studies of the biological factors involved in abnormal chromosome segregation in mammalian germ cells.

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#### MOUSE MUTAGENESIS

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#### Mutation Induction in Zygotes

The first part of the zygote period may be regarded as the final germ-cell stage, since the parental genomes are still separate. We showed some time ago that, at certain intervals after sperm entry, the mouse zygote is extremely sensitive to radiation-induced clastogenic damage.

More recently, W. M. Generoso of our Section has demonstrated the high sensitivity of certain zygote stages to chemical induction of varied congenital anomalies. We are now investigating the sensitivity of zygotes to the induction of gene mutations, using ethylnitrosourea, ENU (which is known to induce primarily intragenic lesions), in a modified specific-locus test. Zygotes containing one parental genome with a number of recessive markers, and the other parental genome with the corresponding wild-type alleles are exposed to ENU by injecting recently copulated females at one of various intervals after the end of a 45-min mating period. To date, about 3000 mice resulting from exposed zygotes, and about 2000 controls have been examined.

While ENU exposure caused prenatal death of a large number of individual conceptuses, the proportion of copulations resulting in litters was not reduced by treatment (except at the highest dose used) when the multiple-recessive parent was the male; it was, however, low in the reciprocal cross. The bulk of the mutation-rate data thus far are therefore derived from mutagenized maternal genomes. Two features of the results are striking: the very high mutation rate and the high proportion of mosaics among mutants. At the most sensitive zygote stage, the mutation rate is almost one order of magnitude greater than that induced by the same dose of ENU in spermatogonial stem cells (in which ENU is considered to be a supermutagen). If ENU is injected at a time when the second meiotic division of oocyte (triggered by sperm entry) is being completed, the mutation rate is 7-8 times higher than when exposure occurs 2.5-3 h later (pronuclear formation).

Of 11 mutants, 8 have been mosaics, and, on the basis of what is known about mouse development, some or all of the whole-body mutants too could have developed from mosaic blastocysts. Genetic tests on several of the overt mosaics have confirmed germline involvement and have ruled out chromosome misassortment at cleavage as a cause of the mosaicism. Some of the results indicate that mutations are induced in one DNA strand of the haploid maternal chromosome; but the possibility of premutational change followed by copy error is not ruled out. Since somatic/germline mosaics can provide tools for studies of cell line lineage, gene action, cell selection, and other developmental mechanisms, the ENU treatment of zygotes could provide a method for generating such tools.

#### **Marked Differences Between Chemicals in Relative Sensitivities of Various Germ-Cell Stages; a Super-Super Mutagen is Discovered**

A concerted effort is under way to enlarge the data base for mammalian germline mutagenesis results which can be used as standards against which data from other test systems are compared in order to assess the degree to which short-term tests are capable of predicting genetic hazards in mammals (and thus presumably in man). All spermatogenic stages, from spermatogonial stem cells to mature spermatozoa are being separately assayed for specific-locus mutations. In the past two years, we have added procedures to our protocol that will yield accurate comparative productivity results, which can be interpreted with regard to

presumptive induction of dominant lethals and/or germ-cell cytotoxicity, thus adding these outcomes to gene-mutation-rate data.

Vastly different patterns of mutagenesis and germ-cell killing were observed among three chemicals studied since the time of the last report. (1) Acrylamide, a chemical whose mutagenic properties have become apparent only in mammalian tests, causes extreme productivity reductions, resulting from dominant lethality (i.e., clastogenic damage) in matings derived from exposed mature sperm, and lesser, but still major, reductions in matings derived from exposed mature spermatids. The specific-locus mutation rate in these latter cells is about 25 times the historical-control rate, and about 12 times the rate for exposed earlier spermatogenic stages (exclusive of stem cells). [The mutation rate in mature sperm is not measurable because the extreme dominant lethality reduced surviving offspring to about 2% of normal.] In contrast to the major mutagenicity in poststem-cell stages, no mutations were found in spermatogonial stem cells, this result probably not being due to cell selection since there is no evidence for stem-cell killing. (2) Adriamycin is a chemical used in cancer chemotherapy. Its productivity effects follow a totally different pattern from that found with acrylamide. Even at the higher dose used by us, there is no evidence for cell killing or dominant lethality in spermatozoa, spermatids, and late spermatocytes. Major cytotoxic effects are produced in differentiating spermatogonia, particularly the early ones, and in early spermatocytes. The higher dose tested by us also produced very severe stem-cell killing, the lower dose only mild stem-cell killing. Mutation-rate data were negative both for spermatogonial stem cells and poststem-cell stages. (3) For chlorambucil, another chemotherapeutic chemical, the productivity pattern is totally unlike that produced by either of the other two chemicals described. Late spermatids are not affected, and early spermatocytes only slightly so. An extremely severe effect occurs in early spermatids, an only slightly less severe one in early differentiating spermatogonia (late differentiating gonidia being somewhat less sensitive) and in late spermatocytes, and a mild effect in mature sperm. The finding for sperm is probably entirely the result of dominant lethals, and that for early spermatids and late spermatocytes at least partially so. On the other hand, the effect seen when differentiating spermatogonia are exposed appears largely due to cytotoxicity.

A striking finding is that chlorambucil may be the most effective germ-cell mutagen studied to date -- even better than ethylnitrosourea in certain germ-cell stages. From exposure of early spermatids, the yield of mutations after only 10 mg/kg is about one per 100 offspring. The mutation rate is also quite high in other postspermatogonial stages, as well as in spermatogonial stem cells. There are indications from the characteristics of the mutants that chlorambucil may be primarily an inducer of deletions and other gross lesions. In view of the utility of deletions for molecular-genetic studies, chlorambucil provides a valuable new research tool.

## Exploration of Factors that Affect Mutation Rate

Numerous complex factors can affect the pathway between exposure of an individual to a mutagen and the expression of a mutation in his offspring. Identification of such factors and investigation of their effect on mutation yield are important for evaluation of genetic risk. Our discovery of an effective mammalian mutagen, ethylnitrosourea (ENU), made such studies feasible, and several have been carried out in the past few years. During the current reporting period, the following experiments were completed or in progress.

(1) **Exposure of very young males.** Because it is known that the frequency of spermatogonia in S phase is greater during the first 18 days postnatally than in adult males, we compared mutation rate in 12- to 14-day-old males with that in 10- to 12-week-old males. The ratio of ENU-induced rates was 1.5. Though the difference was not statistically significant, it is in the direction supporting the view that ENU is most effective at the time of DNA replication.

(2) **Exposure of embryonic males.** Pregnant females were injected with ENU on day 12.5 or 13.5 of gestation, and their sons were raised to maturity and mated for a mutation-rate experiment. In close to 15,000 offspring of the embryonically exposed males, the frequency of mutants was almost twice as high as that observed among offspring of adult males exposed to the same dose. Though the difference is, to date, not statistically significant, the results indicate that male germ cells of embryos are at least as sensitive to ENU mutagenesis as are those of adults, and could be more so.

(3) **Exposure of embryonic females.** Because the oocytes of adult females, which have varying numbers of associated follicle cells, are refractory to many mutagens, it seemed of interest to test mutation rate in oocytes that are not yet associated with follicle cells. Daughters of females injected with ENU on day 12.5 or 13.5 of gestation were raised to maturity and mated for mutation-rate studies. Unfortunately, ENU exposure at these stages appears to be highly detrimental to the survival of female germ cells, resulting in such major reductions in number of offspring as to make mutation-rate determinations unfeasible.

(4) **Dose repetition.** Our earlier experiments had shown that fractionation of a 100 mg/kg dose of ENU into 10 or 5 mg/kg fractions greatly decreased mutation rate. This result, combined with molecular dosimetry studies and the finding that the dose-effect curve for mutations drops below linear at 50 mg/kg and lower doses, led us to propose repair of ENU-induced lesions in stem-cell DNA. To determine whether doses above the levels at which repair occurs are additive, we exposed males to four fractions of 100 mg/kg each at weekly intervals. Additivity was demonstrated, with the total spermatogonial stem-cell mutation rate being higher than  $1 \times 10^{-3}$  per locus. This is by far the highest induced rate of presumed intragenic mutations known for any agent and becomes of great practical value for experiments requiring the "manufacture" of mutations,

such as our saturation mutagenesis study being done in conjunction with molecular analyses of specific chromosomal regions (see report by, Molecular Genetics Group).

(5) **Pretreatment with MNU.** If an O<sup>6</sup>-G alkyl transferase (which has been identified in various types of cells) is responsible for the repair of ENU-induced premutational lesions (ethyl adducts) in mouse germ cells, and if this transferase is more effective in removing methyl than ethyl groups, it may be possible to reduce the amount of transferase available after ENU exposure by pretreating with MNU (without intervening cell division). An experiment of this type was completed (ca 12,000 offspring) involving a 6 h interval between MNU and ENU exposures of spermatogonial stem cells. The pretreatment by MNU failed to increase the ENU-induced mutation rate. The results may indicate that there are different transferases for methyl and ethyl groups, but alternative interpretations are possible.

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#### ORGANISMIC EFFECTS

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#### Dominant Skeletal Mutations

The direct method for estimating genetic risk to humans from radiation is based largely on results from our experimental induction of dominant skeletal mutations. The main emphasis of our Group continues to be the study of the induction and nature of such mutations. Having in the past proved transmissibility of F<sub>1</sub> effects to later generations, a major

subsequent effort has been to develop and improve "non-breeding-test" (NBT) methods for identifying dominant mutations on the basis of morphology alone. Although one cannot then be certain that any given abnormality is caused by a mutation, a significant difference in frequency between treated and control groups indicates induction of dominant mutations. If transmission had to be demonstrated before an abnormality could be counted, several types of mutations would be missed. These include mutations that have severe effects in only a small proportion of the animals carrying them and mutations that cause their carriers to be sterile or to die before they can be genetically tested. Some of the organismically most deleterious mutations fall into the last category.

We have shown the chemical ethylnitrosourea (ENU) to be very effective in inducing dominant skeletal mutations, a finding that was somewhat surprising in view of the fact that this chemical induces mainly base-pair substitutions, while dominant damage is sometimes thought of being associated primarily with larger lesions, such as multilocus deletions. To further improve the NBT methods and to learn more about ENU at the same time, we are presently conducting a moderately large experiment that applies both NBT and breeding-test methods. Males were injected with four, weekly 100-mg/kg exposures of ENU, and the frequencies of presumed dominant skeletal mutations are being determined for the exposed group and the concurrent control. Our special interest is in studying the evidence of transmissibility for these various effects.

An important emphasis in the skeletal work is on mutations with incomplete penetrance (i.e., those that are not expressed in all carriers). Such mutations are of great concern, because they relate to the irregularly inherited disorders, which account for the great bulk of serious human genetic disorders. Many dominant skeletal mutations exhibit incomplete penetrance and variable expressivity.

Work also continues on searching for mutations that are models of specific dominant genetic disorders in humans. The array of effects produced by our cleidocranial dysplasia mutation, for example, is very similar to that seen in humans carrying a mutation of the same name.

Because the skeletal methods provide a way of detecting effects of altered genetic material in a sizeable part of the genome, we will use these techniques to try to identify mutations in transgenic mice, as well as in mice exposed to certain mutagens soon after fertilization.

### **Stunted Growth**

This year we were able to demonstrate that stunted growth can be a useful indicator for dominant genetic damage. Highly significant effects could be demonstrated in samples of just a few hundred offspring. In this experiment, first-generation offspring of males exposed to four weekly exposures of 100 mg/kg of ENU were weighed when  $79 \pm 4$  days of age. Mice were considered to have stunted growth if they were less than, or equal to, two standard deviations below the mean weight at this age for litters

having the same number of offspring when they were weaned. Using these criteria, the fraction of mice with stunted growth was 49/492 in the experimental group and 16/521 in the control, the frequency of stunted growth being significantly higher in the experimental group ( $P < 0.00001$ ), and the induced frequency 6.9%.

The importance of basing calculations on litters having the same number of animals at weaning became apparent when we discovered that even at 79 days of age there is a strong, and statistically significant, negative correlation between litter-size and weight (such a correlation had earlier been known for weaning-age animals). Moreover, W. L. Russell and P. R. Hunsicker of our Section have demonstrated that the  $4 \times 100$  mg/kg ENU treatment induces an 11% litter-size reduction (LSR). Since 11% of  $F_1$  descendants die between conception and 4 weeks of age, the experimental animals, on the average, came from smaller litters, and the litter-size reduction would have partially obscured the effect on body size. Parents of both sexes were randomized between the experimental and control groups to decrease the chances that preexisting mutations would confound the results. The same experiment produced the findings on LSR and on stunted growth, and some of the first-generation offspring are now being evaluated for dominant skeletal mutations.

#### **Critical Review of Experiments on Induction of Dominant Mutations**

A major undertaking this past year was to take a critical overview at the whole area of dominant-mutation induction and risk estimation in order to identify gaps in our knowledge and profitable directions for future research. Reported work on skeletal abnormalities, cataracts, externally visible changes, litter-size reduction, congenital malformations (detected in late pregnancy), stunted growth, shortened life span, tumors, and effects on behavior was critically reviewed.

Because certain types of information essential for the improvement of genetic risk estimates can be obtained only by the study of induction of dominant mutations (specifically, the nature and magnitude of organismic effects in the first descendant generation), we have proposed that several different endpoints (e.g., certain types of skeletal malformations and cataracts, litter-size reduction, stunted growth, and perhaps congenital malformations) be studied in the same experiment. Such a test might detect mutations in hundreds of genetic loci, and, with some refinements in methodology, might eventually be able to serve as a detector of the mutagenic potential of an agent, as well as supplying results that can readily be related to genetic risk estimates. The experiment with  $4 \times 100$  mg/kg ENU, which has yielded results on stunted growth and reduced survival (see above) and is now yielding data on skeletal mutations, is a first step in the exploration of the utility of multiple-indicator tests.

### Computer Analysis of Large Data Sets

Results from many of the specific-locus experiments performed over decades by the Mammalian Genetics Section have now been entered into a data-base management system on a mainframe computer. Ongoing specific-locus experiments are also being entered into this system. The data are now amenable to analyses that would not have been feasible without computers. For example, one analysis on radiation-induced small litter-size reductions, required the calculation of the age of the mother at the birth of each litter for 158,490 litters. Several additional computer analyses are in progress, including one on radiation-induced death resulting from mutations acting dominantly between birth and three weeks of age and another on sex ratios in offspring of irradiated and control mice. Our group has been active in performing such analyses, in developing applications on the mainframe computer, and, more recently, in improving the efficiency of using personal computers for data collection and for transferring data to the mainframe computer. Among programs that we have recently written for personal computers is one that speeds up the performance of the saturation mutagenesis experiment (see Molecular Genetics report) and one that permits us to make more informed decisions about which sublines to cut back to when maintaining large inbred strains.

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### GERM-CELL DNA/PROTEINS

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The purpose of our research is to develop an understanding of the molecular processes that lead to the induction of mutations in mammalian germ cells, using the mouse (both males and females) as a model system. A better understanding of these mutational processes at the molecular level

is important for establishing reasonable guidelines for estimating human genetic risk from exposure to chemicals and radiations in the environment. It is also necessary for developing molecular techniques to monitor human germ cells for potential genetic damage. The work can be divided into several interrelated areas of research: (1) *Molecular dosimetry* to measure the amounts and types of interaction between chemical agents and genetically significant targets within the germ cells. This dosimetry gives valuable information on the relationship between exposures of the whole animal to a mutagen and the amount of mutagen actually reaching the germ cells. (2) *In vivo DNA repair* studies that give important information, in a relatively short period of time, on the extent to which a chemical agent is able to reach the DNA of mammalian germ cells and produce "repairable" lesions. Differential DNA-repair capabilities of various germ-cell stages exposed to the same mutagen can also be measured by means of these studies. (3) *Measurement of DNA strand breakage* in the germ cells following mutagen exposure, using alkaline elution techniques to recover broken pieces of DNA.

### **Molecular Dosimetry**

Molecular dosimetry using radioactively labeled chemical mutagens has been an excellent method to reveal the molecular events occurring within germ cells after treatment with mutagens. The procedure is extremely sensitive and can be used to measure binding of chemical agents to germ cells and to germ-cell DNA at exposure levels that are orders of magnitude lower than those necessary to produce a statistically significant genetic effect. Typically, as little as 1 lesion in  $10^8$  deoxynucleotides can be detected with this method. In addition to procedures that make use of labeled mutagens, other techniques for measuring DNA adducts are being explored.

We have obtained further evidence that protamines, as well as DNA, are important molecular targets for chemical attack that may lead to chromosome aberrations. (The sperm protamines of both mouse and man are simple proteins that are intimately associated with DNA and found only in late-spermatid and mature-sperm stages). Recent studies using low-level inhalation exposures of ethylene oxide (EtO) showed large increases in binding to late spermatids and early spermatozoa. Alkylation of DNA within these stages averaged only about 20 DNA adducts per cell for each part-per-million-hour (ppm-h) of exposure. The remaining 6000 adducts per cell per ppm-h of exposure were found to be bound to protamine. Furthermore, the temporal pattern of EtO binding to protamine paralleled the pattern of genetic damage produced by EtO in the spermiogenic stages, while DNA alkylation showed no such correlation.

As our molecular dosimetry data are combined with other genetic and cytogenetic data for the same chemical agents, we will continue to learn more about the relationship between the extent of chemical damage in the germ cells and the amount of genetic damage expected at realistic exposure levels for humans.

## DNA Repair

DNA repair can occur in a number of spermatogenic stages but not in the most mature ones. The DNA repair response of germ cells to a given chemical mutagen can vary several-fold among different mouse strains. Our observation of DNA repair in mammalian germ cells after mutagen exposure led us to develop a test for unscheduled DNA synthesis (UDS) that has been used for the screening of potential mutagens because it is fast, sensitive, and rather inexpensive.

We have shown that the route of chemical exposure can affect the level of UDS measured in the germ cells. Thus, intraperitoneal injections of chemicals such as methyl methanesulfonate give a higher UDS response in germ cells than does exposure by gavage, presumably because the former route of exposure can deliver more chemical to the germ cells than the latter can.

The UDS response of the germ cells has also been shown to be a function of the rate at which the chemical is administered. Thus, an exposure of 1800 ppm of EtO for 1 h gives a UDS response in early spermatids that is about 3 times greater than an exposure of 450 ppm given for 4 h. Such dose-rate effects can alter genetic risk estimates when one is extrapolating from high to low chemical concentrations.

Recent studies with acrylamide have indicated unequivocally that this chemical is able to produce DNA lesions in early spermatids. However, the DNA repair response is delayed, with the maximum response occurring 6 h after acrylamide exposure. No other chemical studied to date has shown a delayed UDS response of more than 1 h.

## Alkaline Elution

To further assess the damage produced in mammalian germ cells using model chemicals, we have developed an alkaline elution procedure to measure DNA strand breaks in the germ cells. The procedure involves lysis of the germ cells after treatment with the test chemical, followed by separation of the DNA double helix using a strong base. Any small pieces of single-stranded DNA resulting from breakage by the test chemical will rapidly pass through a filter, while normal-sized DNA will take much longer to pass through the same filter. The pattern of DNA breakage in mouse spermiogenic stages exposed to certain mutagens such as methyl methanesulfonate and EtO has been found to parallel the pattern of sperm-head alkylation and the pattern of induced dominant lethals and heritable translocations.

Using centrifugal elutriation procedures to isolate specific germ-cell stages, alkaline elution studies have been carried out on pachytene spermatocytes and early spermatids after exposure to EtO and X rays. The results show a great difference in the way germ cells handle the repair of DNA breaks induced by each agent. For X rays, all DNA breaks appear to be repaired within about 1 h after exposure, while single-strand breaks can

still be detected in the germ cells as long as 3 days after exposure to EtO.

This alkaline elution procedure may prove especially useful in measuring accumulation and/or persistence of DNA strand breaks in spermiogenic stages in which no DNA repair is observed after mutagen treatment. It is possible that this method of detecting DNA damage in mouse sperm may eventually prove applicable for monitoring DNA damage in human sperm.

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## Human Genetics Section

### Section Overview - R. J. Preston

The activities of the Section are integrated to provide a composite picture of the potential genetic and somatic effects of radiation and chemical agents to man. This goal is addressed by a research program that is designed to provide an understanding of normal cellular processes (e.g., DNA replication, gene expression) and the consequences of perturbations of these. While this goal is the one that we have had for some time, we continue to utilize new approaches to reach it, so that we take advantage of new techniques that we or others develop.

The advent of molecular techniques has made it quite feasible to study gene mutations and chromosomal alterations at the level of the DNA sequence and to understand more completely cellular processes in normal and mutant cells. The research of the Section is taking advantage of these techniques in order to address specific problems as described below. In addition, we have used a variety of whole animal and cellular model systems to obtain information that it is anticipated will be used to extrapolate to man. Some examples are presented here to illustrate these points; more detailed descriptions can be found in the individual research reports that follow.

(1) Mouse mutant stocks have been developed for a variety of hemoglobin abnormalities characterized as having altered gene products or expression. One of these, a  $\beta$ -thalassemic mouse that serves as a unique model for human thalassemia, has been extensively studied in Oak Ridge and in many other laboratories. It is anticipated that this model system can be used to determine the feasibility of gene therapy, employing the normal human gene for reverting the mouse mutant phenotype.

(2) Ciliated protozoa possess two distinct nuclear types, the micronucleus and macronucleus. The latter exhibits a localization of DNA synthesis to a replication band that migrates along the nucleus during the S phase. This provides an excellent model for studying the details of DNA replication. For example, by employing a macronuclear DNA expression library, DNA clones have been identified that code for replication band specific proteins. Also, an *in vitro* assay for DNA replication by isolated macronuclei has been developed.

(3) Restriction endonucleases have been utilized to produce chromosome alterations in Chinese hamster and human lymphoblastoid cells. This technique allows us to produce particular types of DNA double-strand break at specific recognition sequences. By choosing appropriate enzymes it is quite feasible to produce alterations at specific locations that might be of genetic significance. We can also use this approach to determine the steps between initial DNA damage and its conversion into

chromosome aberrations, and if there are potential "hot spots" for aberration induction.

(4) Ultraviolet light induces a variety of DNA photoproducts; the most important with regard to cell transformation and mutation induction generally regarded as being the thymine dimer [(5-5,6-6) cyclobutane dimer]. Recent studies have indicated that the (6,4) pyrimidine-pyrimidone dimer [(6,4) photoproduct] could also be of significance; the lack or decreased rate of repair of this product in xeroderma pigmentosum cells (especially the variant) could be the cause of their increased UV-light sensitivity. A detailed analysis of DNA repair in Chinese hamster UV-sensitive cell lines and the same cell lines transfected with human DNA repair genes could provide confirmation for the role of the 6,4-product.

These studies and others described in the following sections make it possible to determine the relationship between the induction of specific DNA damages induced by radiation and chemical agents and the production of gene mutations and chromosomal alterations. In addition, it will be possible to determine how misrepair or failure to repair DNA damage is involved in the process of mutation induction and replication of damaged templates can result in mutations. The importance of the non-random induction of DNA damage and/or repair on the spectrum of gene mutations and chromosomal alterations is being considered. If the spectrum is clearly different for "spontaneous" and induced mutants, and different for each inducing agent or class of agents, it would be possible to accurately classify a mutation on the basis of its origin. This would be of considerable importance for assessing the risk of exposure to radiation or chemical agents in man.

#### MOLECULAR CYTOGENETICS

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The various research projects of the Molecular Cytogenetics Group are designed to explore three general areas: the mechanism of induction of chromosome alterations and mutations by ionizing radiations and chemical agents; the likelihood that chromosome alterations and mutations are induced non-randomly; the role of specific chromosomal alterations and changes in gene expression in cell transformation and tumor progression. There is evidence that specific chromosome alterations and/or amplification and mutation of oncogenes are involved in the induction or

progression of some tumors, most specifically leukemias and lymphomas. Our studies have concentrated on solid tumors to determine if similar genetic alterations are involved in their development. In addition, in order to estimate the relative risks of radiations and chemical agents for the induction of genetic and carcinogenic outcomes in man, it is necessary not only to determine the mechanism of induction, but also to consider the possibility of whether or not there are hot spots for the induction of chromosome alterations and mutations. Furthermore, if hot spots exist, are they dependent on the mutagenic agent? Current research in these areas is presented below.

### **Induction of Chromosome Aberrations by Restriction Endonucleases**

In order to provide a simple model system for determining how specific types of DNA damage might be converted into chromosome aberrations, we have utilized restriction enzymes to induce the damage. The choice of a particular enzyme allows for the induction of blunt-end double breaks or cohesive-end double-strand breaks with different lengths of overlap. In addition, the recognition sequence for a particular enzyme should make it possible to estimate the relative cutting frequency in the genome, and thus the amount of damage should be variable from frequent to rare.

We developed a technique based upon the osmolytic shock of pinocytic vesicles to introduce the enzymes into Chinese hamster fibroblast cells and human lymphoblastoid cells. This is a reliable method, and of particular importance there is no decrease in cellular viability.

Chromosome aberrations can be produced from both blunt-end and cohesive-ended double-strand breaks, with the frequency being proportional to the calculated cutting frequency, and not to the type of cut-end structure. Thus it appeared feasible that specific chromosome aberrations could be produced by enzymes that cut very infrequently. This proved to be the case with NotI (a rare-cutting enzyme) that produced a low frequency of aberrations at only some four sites in Chinese hamster cells. This should make it possible to design a method by which specific aberrations can be induced, for example at fragile sites.

In order to develop a model for radiation-induced aberrations, interaction experiments were conducted with pairs of restriction enzymes that produced either blunt- or cohesive-ends. The aberration frequencies were considerably above additivity when the enzyme pairs produced either blunt-ends or cohesive-ends of two bases in length. However, if the cohesive-ends were for a four-base overlap, interaction did not occur. This suggests that double-strand breaks induced by X rays (generally more than four-bases) are a minor component in aberration production, whereas the double-strand breaks induced by higher LET radiations are likely to be a major component.

### Characterization of Chinese Hamster Radiation-Sensitive Mutant Cells and a Comparison with Human Mutant Cells

Chinese hamster ovary (CHO) cells were selected for resistance to ara-C, an inhibitor of DNA repair resynthesis. Selection was performed at a concentration of  $5 \times 10^{-5}$  M. X-ray cell survival curves of one of these mutants showed that there was a tenfold sensitivity of the mutant compared to wild-type cells. If ara-C was given for 3 h post X irradiation, the mutant exhibited no change in the number of surviving cells compared to that following X irradiation without an ara-C post-treatment. The wild-type cells show an increased sensitivity with a 3 h post-X-ray ara-C treatment, similar to that for the mutant. These observations are very similar to those for AT cells compared to normal human cells (discussed below). The frequency of X-ray-induced chromosome aberrations is consistently higher in the mutant compared to wild-type cells. Our intention is to perform complementation studies between CHO mutants and AT cells to determine if the defect in the CHO mutant is the same as that in AT cells. We also propose to carry out DNA transfection experiments with human DNA to attempt to "revert" the mutant and then to isolate and characterize the human gene(s) involved.

Further characterization of this ara-C resistant/X-ray-sensitive mutant CHO cell supports our hypothesis that it is similar to the AT cell lines. A measurement of the mitotic index following X irradiation shows that it does not undergo the pre-mitotic delay exhibited by wild-type CHO cells -- this is the same result as is found for AT cells versus normal human cells. In addition, the mutant CHO cell does not show a radiation-induced delay of entry into the S-phase; wild-type cells do. Again, this result is the same as for AT cells compared to normal human cells.

Parallel studies to those reported above have been carried out with AT5BI and normal human fibroblasts. The AT cells are more sensitive to X-ray-induced cell killing than normal cells (5- to 10-fold), but if the cells are treated for 3 h post-irradiation with ara-C, the mutant has the same sensitivity as with X rays alone. In contrast, the normal cells show an increased sensitivity. The normal cells have a similar sensitivity as the mutant with an ara-C post-irradiation treatment. Because of the role of ara-C as a repair resynthesis inhibitor, AT cells possibly have a defect in this step of excision repair. Similar results were observed for chromosome aberration induction. Normal cells are less sensitive to aberration induction by X rays in  $G_2$  than AT cells, but with a 2 h ara-C treatment after X rays the sensitivity of AT cells does not change. The aberration frequency in the normal cells increases to a level similar to that for the AT cells. These studies will be continued to obtain additional information on the defect in AT cells that makes them radiation-sensitive. AT cells will also be used to study the role of repair (or misrepair) in the induction of chromosome aberrations.

### **Comparison of the Frequency of Chromosomal Alterations Analyzed at the Molecular and Microscopic Levels**

From our preliminary studies designed to determine X-ray dose response curves for chromosome aberrations in the parent lymphoblastoid line (GM606) it was apparent that there was considerable cell heterogeneity. In order to overcome this, we isolated several single cell clones, three of which have been characterized karyotypically and for sensitivity to aberration induction. Two of the clones (W1 and W2) have a normal chromosome number of 46, and each contains a single reciprocal translocation; the third (W3) contains 48 chromosomes with additional Chromosomes No. 11 and 13. The aneuploid clone (W3) was approximately three times as sensitive as the other two to the induction of chromosome aberrations by X rays. In addition, this increase for  $G_1$  irradiated cells was predominantly for chromatid-type aberrations, as has been observed for cells from persons with ataxia telangiectasia. Further studies are in progress to identify the reasons for the increased sensitivity.

### **The Role of Recombinational Processes in the Production of Chromosome Aberrations**

Data obtained in our laboratory and in others suggest that chromosome aberrations (some or all) are produced by a recombinational process. We have initiated studies to test this hypothesis as it is of considerable importance to determine if such processes are responsible for aberrations, since it would provide a viable explanation of how specific chromosomal alterations could be produced. The role of specific chromosome alterations in the production of tumors and birth defects is becoming very significant.

Previous studies in our laboratory showed that the frequency of sister chromatid exchanges (SCE) was about five times as high when cells were grown in chlorodeoxyuridine (CldU) compared to growth in bromodeoxyuridine (BrdU). SCE are produced by an intrachromosomal recombination-like process during DNA replication, so it is apparent that this phenomenon might be used to consider the role of recombination in chromosome interchange production. The rationale is that the frequency of chromosome interchanges produced by X irradiation should be higher in cells grown in the presence of CldU compared to BrdU if a recombinational process is involved.

Chinese hamster cells were grown in a range of concentrations of CldU or BrdU irradiated with X rays, and fixed 3 h later - the sampled cells would have been irradiated in  $G_2$ . The frequency of aberrations in unirradiated cells was considerably higher in cells grown in CldU than those containing BrdU, in agreement with our earlier observations for SCE. The frequency of chromatid deletions was higher in cells containing BrdU; that might be expected from the fact that BrdU-substituted is more sensitive to DNA damage by X rays than CldU-substituted DNA. However, the frequency of chromatid interchanges is significantly higher in CldU-substituted cells at higher base analog concentrations. This observation

provides preliminary evidence that a recombinational process, enhanced when CldU is present in the repairing DNA, could be involved in interchange production. Preliminary results for cells irradiated with neutrons or treated with bleomycin generally support this hypothesis. However, more data are clearly needed before definitive conclusions can be drawn.

### **The Relationship Between the Induction of Specific Chromosome and Gene Alterations in Tumor Induction and/or Progression**

In collaboration with W. K. Yang and R. L. Ullrich, we have been analyzing karyotypic and oncogene changes in the production of mammary tumors. Ionizing radiation causes genetic alterations and induces tumors in animals. This correlation suggests that genetic alterations may be produced as a direct result of radiation exposure and that these genetic alterations may subsequently lead to secondary tumorigenic events that are associated with the neoplastic progression. The objective of our study was to elucidate the relationship between neoplastic progression and non-random chromosomal and oncogene alterations involved in radiation carcinogenesis. Mammary epithelial cell lines derived from a radiation-exposed BALB/c mouse were assayed for non-random chromosomal alterations and oncogene activation with neoplastic progression *in vitro*. Chromosomal analyses suggest that homogeneously staining regions (HSRs), double minutes (DMs), and specific metacentric indicator chromosomes (frequently isochromosome 6) (MICs) are associated with the later stages of the neoplastic progression of these cells. Analyses of *in vivo* tumor metaphases obtained from *in vitro* cell lines indicate that predominantly near diploid cell populations are associated with tumor formation. Further, Southern and Northern blot analyses indicate a correlation between c-myc overexpression and amplification and the presence of HSRs, DMs, and MICs in these cells. These results suggest that specific, non-random, chromosomal and oncogene alterations are involved in the neoplastic progression of radiation carcinogenesis.

Additional cell lines and single cell clones from these are being developed to determine if these observations are generally associated with mammary tumor development, or are unique to the line studied to date.

A similar type of study is being conducted in collaboration with R. J. M. Fry of the Biology Division. By the time solid tumors are normally observed, many chromosome changes are present. Observing tumors early in development and following chromosome changes with the progression of tumors may help to determine if specific chromosome changes are associated with tumors at different stages of development and if particular chromosome changes are dependent upon the inducing agent. Plastic discs are implanted subcutaneously into mice, resulting in development of sarcomas at the site of the disc. The discs are removed at various times from 34 weeks post-implantation until mature sarcomas are observed. The associated cells are then karyotyped or established in culture for further analysis. Chromosome banding analysis is performed in the karyotyping process. In some cases the animals were treated with

X rays or chemical carcinogens, and again the sarcomas are karyotyped. In summary, based on a large number of observations, the chromosome numbers vary from 52 to 200 for the sarcomas analyzed, with much more variation among tumors than within a particular tumor. The cell "nubs" that initially form around the plastic discs, prior to any apparent preneoplastic change, consist of cells with low chromosome numbers - close to the normal 40. A variety of chromosome changes have been identified and although no consistent pattern of change has been observed, there appear to be some changes that occur rather frequently: extra copies of chromosome numbers 10, 16, and 19 in 60% of the sarcomas.

In addition, double minutes, indicative of gene amplification, are present in many tumors. Initial attempts to characterize the particular gene(s) amplified have not generated any positive results. To date we have shown that there is no apparent major amplification of the oncogenes *myc*, *K-ras*, *src*, *fos*, or *myb*. We are currently determining if the multiple drug resistance gene is amplified in these sarcomas, as has been observed in studies in other laboratories.

As each of these studies progresses, it is anticipated that they will provide a composite picture of how chromosome aberrations are produced by radiation and chemical agents, how the proposed mechanism allows for an explanation of the production of specific chromosome and gene alterations (hot spots), and whether or not such specific alterations play a role in the induction of adverse genetic and somatic health effects.

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### MAMMALIAN BIOCHEMICAL GENETICS

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The purpose of our program is to determine the mutagenic and health effects of irradiation, chemicals, and viruses in mice. Although mice are used as experimental animals, the data obtained are analyzed with respect to potential human hazards. Techniques of molecular biology are needed to study the organization and regulation of genes and the structure and function of gene products. The molecular and biochemical basis for naturally occurring and induced mutations and the physiological and pathological consequences of several genetic lesions are being studied. Mutants that represent animal models of inherited human diseases are of special interest. Animal models of human alpha- and beta-thalassemia are being inbred to develop and test alternative techniques of gene therapy that might have future applications to the treatment of human thalassemia. B10.F mice shed an endogenous ecotropic virus that integrates in the DNA of lymphatic cells. Recombinant DNA, flow cytometry, and immunological methods are being used to determine the mode of viral transmission and the nature of the immunodeficiency observed in B10.F mice. Summaries of our recent studies are presented below.

#### Organization and Regulation of the Duplicated Alpha-Globin Genes in Mice

The mouse alpha-globin complex contains three genes (x - a1 - a2) arranged in the 5' → 3' direction. The embryonic x-globin gene is expressed only in the nucleated erythrocytes that arise in the visceral yolk sac and the duplicated adult a1 and a2 genes are both expressed in the nucleated erythrocytes of yolk sac origin and in the non-nucleated erythrocytes that differentiate in the fetal liver, spleen and bone marrow. We have isolated the nucleated and non-nucleated erythrocytes by centrifugal elutriation to show that the tandem alpha-globin genes are expressed at different levels in nucleated erythrocytes derived from the yolk sac compared to the non-nucleated erythrocytes derived from the fetal liver or bone marrow. Studies are in progress to compare the nucleotide sequences of the structural genes and flanking sequences in attempts to

identify nucleotides that might regulate the differential expression of these genes in different tissues at different times during development.

### Skive Mice have a Unique Alpha Chain

The hemoglobin chain haplotypes of several stocks of wild mice from throughout the world have been assigned on the basis of isoelectric focusing patterns. The amino acid sequences of the alpha chains in hemoglobins from mice with the  $Hba^{w2}$ ,  $Hba^{w3}$  and  $Hba^{w4}$  haplotypes were determined to establish whether the tentative alpha-chain assignments were correct. As predicted, hemoglobin from "Centreville," Maryland, *Mus musculus domesticus* ( $Hba^{w2}$ ) contains equal amounts of alpha-chains 1 and 3, and hemoglobin from "Czech" *Mus musculus musculus* ( $Hba^{w4}$ ) contains equal amounts of alpha-chains 3 and 4. We found, however, that hemoglobin from "Skive" Danish *Mus musculus musculus* ( $Hba^{w3}$ ) is comprised of about one-third alpha-chain 2 plus a greater amount of a unique alpha chain that had not been described previously. This unique alpha chain, which we have designated chain 7, has isoleucine rather than valine at position 62 and serine rather than asparagine at position 68. The alpha-globin genes of "Skive" mice are being cloned for analysis of the nucleotide substitutions in the structural genes that code for chain 7 and nucleotide sequences that regulate the different levels of expression of the genes that code for alpha-globin chains 2 and 7 in "Skive" mice.

### Mutations that Cause Mouse Hemoglobins to be Unstable

Some anemias in humans are caused by unstable forms of hemoglobin. We reported that the hemoglobin of mice with the  $Hbb^{s2}$  mutation, which has a glutamic acid for valine substitution at position 60 in the beta-major chain, is unstable under conditions of mild denaturation in 20% isopropanol. Using the same method, we found that the hemoglobin of mice with the  $Hba^{g2}$  mutation, which has a leucine for histidine substitution at position 89 in the alpha chain, is stable in 20% isopropanol. Matings were made to produce mice that carry both mutations. Mouse hemoglobin comprised of alpha- and beta-globin chains with both of these amino acid substitutions has the instability of hemoglobin with only beta globin amino acid substitutions. Although hemoglobins with these amino acid substitutions are not noticeably unstable under normal physiological conditions, mice that were homozygous for both mutations died earlier than mice that were homozygous for either mutation.

We have shown that the oxygen-binding affinities of hemoglobins from mice with  $Hba^{g2}$  ( $P_{50} = 32$  mmHg) and  $Hbb^{s2}$  ( $P_{50} = 31$  mmHg) is higher (has a lower  $P_{50}$  value) than for hemoglobin from normal mice ( $P_{50} = 42$  mmHg). These specific amino acid substitutions alter the heme-binding sites and increase the strength of the noncovalent association of oxygen with heme. Hemoglobin with a high affinity for oxygen does not transport oxygen efficiently. The poorer survival of mice doubly homozygous for the  $Hba^{g2}$  and  $Hbb^{s2}$  mutations may be associated with the low  $P_{50}$  value (25 mm of Hg) of their hemoglobin.

### Hematological Indices of Beta-Thalassemic Mice

Two congenic strains of beta-thalassemic mice are being developed. Their natural hematology is being compared to that of normal mice so that the baseline values can be used in the future to measure the efficacy of alternative methods of gene therapy to correct the deficient expression of beta globin in experimental mice. The average lifespan of red cells from normal mice is 40 days and from beta-thalassemic mice is only 16 days. Red cell homeostasis is maintained through production of  $3.6 \times 10^8$  erythrocytes per day in normal adult mice and  $11.6 \times 10^8$  erythrocytes per day in adult beta-thalassemic mice. We have determined that the threefold increase in red cell production in beta-thalassemic mice is sustained by increased extramedullary hematopoiesis. The cellularity of the spleen is increased threefold; it contains 3.5 times as many pluripotent colony-forming stem cells and committed erythroid burst-forming cells and 150 times as many terminally differentiating erythroid colony-forming stem cells. The spleen of human thalassemic patients also becomes enlarged, but it is usually removed. The spleen has been removed from a large group of beta-thalassemic mice to determine the effects of splenectomy on erythropoiesis. The results of these studies have direct application to human patients where similar experimental studies are difficult or impossible to do. Gene therapy experiments, where retrovirus vectors will be used to introduce mouse or human beta-globin genes into the hematopoietic stem cells of beta-thalassemic mice, will be initiated within the next year.

### Differential Sensitivity of Mouse Strains to Ethylene Oxide

Classical genetic studies have been used to establish that a single pair of recessive genes in SEC/R1, BALB/cJ and SWR/J mice results in sensitivity to ethylene oxide. Kidney lesions, hemorrhage and death occur in mice of the sensitive strains but not in resistant strains, like C57BL/6 mice. Linkage analysis and the strain distribution patterns for genetic markers segregating in CXB/By recombinant inbred strains of mice failed to locate the gene responsible for ethylene oxide sensitivity in approximately 50% of the genome specifically tested.

Calcium oxalate crystals were found in the kidneys of SEC/R1 but not in C57BL/6 mice, so we searched for differences among enzymes that might metabolize ethylene oxide to form oxalic acid. The conversion would be mediated by alcohol dehydrogenases, aldehyde dehydrogenases, aldehyde oxidases and hydroxy acid oxidases. No specific isozyme could be identified that might cause SEC/R1 mice to be sensitive and C57BL/6 mice to be resistant to ethylene oxide. Thus, the biochemical basis for the difference has not been solved.

Dominant lethal studies were undertaken to show that ethylene oxide also has a greater effect on the germ cells of SEC/R1 than C57BL/6 mice. The stages of spermiogenesis most affected by ethylene oxide are the late spermatids and spermatozoa in C57BL/6 mice and the early spermatids and late primary spermatocytes in SEC/R1 mice. Exposure of males to 50 ppm of

ethylene oxide for 6 h a day for 5 days induced both a significant preimplantation and postimplantation loss for SEC/R1 but not for C57BL/6.

#### Effects of a Maternally-Transmitted Ecotropic Retrovirus in Mice

Another major effort in our laboratory involves the characterization of strain B10.F mice. B10.F-H-2<sup>n</sup> is congenic with strain B10-H-2<sup>b</sup>. The former grays earlier, has a shorter lifespan, lower immune responsiveness, and sheds an ecotropic virus that integrates into somatic cell DNA. Histological sections show that the lymph nodes and spleen are often hyperplastic and are characterized by plasmacytosis, inflammatory reactions, and amyloidosis. The viral integration can, but does not always, initiate the formation of lymphomas and reticulum cell sarcomas. The incidence of viral integration, the chromosomal location of the integrated viral sequences, and the structure of the integrated virus are being studied. Integrated viruses commonly cause over-expression of adjacent, specific DNA sequences that initiate tumor cells to grow. Studies on the chromosomal sites of viral integration could provide information on the location of cellular genes capable of transforming cells.

The immune system of B10.F mice is impaired. The immune competence of specific cellular compartments were assessed, and the affected cells and/or the target cell were identified. Immune responses to ConA and PHA (T helper and T suppressor, respectively) were depressed, and response to LPS (B cell) was normal. A FACS Star flow cytometer, located in the University of Tennessee Memorial Research Center, was used to quantify lymphocyte subpopulations that were tagged with monoclonal antibodies specific for cell surface markers. B10.F spleen has 30% fewer Thy 1<sup>+</sup> (T cell antigen) cells, which correlates with the depressed response to ConA. B10.F spleen also has fewer Lyt2<sup>+</sup> (T suppressor) cells. The relationship of the "AIDS-like" reduced immune response and specific viral integration can be determined by examining the DNA of sorted cells, which will require the availability of a FACS Star cell sorter.

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#### THE REPAIR OF HUMAN DNA

J. D. Regan	A. A. Francis
W. L. Carrier	B. G. Stanford

Human cells contain DNA repair systems responsible for the removal of environmentally-induced DNA lesions which are potentially carcinogenic. These DNA lesions may be sunlight-induced DNA photoproducts or chemical adducts produced by environmental carcinogens such as benzo(a)pyrene.

Individuals with the genetic disease xeroderma pigmentosum (XP) have a mutation causing a defect in the DNA repair system. Such individuals suffer from multiple sunlight-induced cancers. Cells from classical XP patients show an inability to excise *cis-syn* cyclobutane pyrimidine dimers from their DNA. As a result of these observations this pyrimidine dimer has been considered a primary candidate for the ultimate ultraviolet-induced carcinogenic lesion in DNA.

Other XP patients, grouped as XP variants (XPV), do excise *cis-syn* pyrimidine dimers but, nevertheless, sustain multiple solar carcinomas. In XPV patients we found a failure to repair another ultraviolet-induced DNA lesion, which is sensitive to 313 nm irradiation and is detected by DNA molecular weight determinations on alkaline sucrose gradients. This lesion is repaired much more rapidly in normal human cells than is the *cis-syn* pyrimidine dimer. The removal of this lesion is temperature dependent in normal cells indicating that an enzymatic reaction is involved. Biophysical experiments on the action spectrum for induction and photolysis suggest that this new lesion may be a 6-4 pyrimidine

pyrimidone. Such products are induced most readily at 254 nm and are most photolyzable at 313 nm. The fact that this lesion is rapidly repaired in normal human cells may indicate a major biological significance even though its incidence in DNA after ultraviolet (UV) is much lower than that of the *cis-syn* dimer. Our current research is concerned with chemical determination of the relationship between the 6-4 product and the photosensitive lesion. We are also assaying for the possible alteration or processing of the lesion within the complementation groups of classical XPs. Even though classical XP cells cannot excise the lesions, there is evidence that they change its properties. This alteration step could be deficient in the XPV.

#### **Cloned Human Repair Genes and UV Sensitive CHO Cells**

Transfection of the cloned human excision repair gene, ERCC-2, to a UV-sensitive Chinese hamster ovary (CHO) (UV5) mutant corrects the pyrimidine dimer repair defect. Normal CHO cells (AA8), a UV-sensitive cell line (UV5), and a UV5 cell line transfected with the cloned human excision repair gene ERCC-2 (5C24-2) were obtained from L. H. Thompson of the Lawrence Livermore Laboratory. We analyzed these cell lines for their ability to excise UV-induced pyrimidine dimers from their DNA. Normal human cells have the ability to excise about  $1 \times 10^6$  pyrimidine dimers in 24 h. Rodent cells (mouse and CHO) are able to repair one-third the amount of human cells. We measured the  $^3\text{H}$ -thymidine containing dimer content of cells by two-dimensional paper chromatography. With improvements in this technique we are able to follow the fate of dimers after very low doses of 254 nm UV-light. UV-5 does not excise dimers. The 5C24-2 cell line excises dimers at the same level as AA8 and demonstrates the efficient function of a human repair gene in the hamster system.

#### **UV-Induced DNA Damage Differs Quantitatively with Cell Type**

Many cellular studies involving DNA damage are concerned with a comparison of sensitivity to UV light. For example, in interspecies comparisons, rodent cells excise pyrimidine dimers at a slower rate than primate cells although their UV sensitivities are similar. This result may be due to preferential repair of actively transcribed genes in rodent cells. While these factors may be important, other considerations are necessary in the comparison of the radiosensitivities of different cell types. The DNA of different cells may receive different amounts of UV light. A good indication of this phenomenon is the dimer content of UV-irradiated cells. We compared the dimer content of irradiated, human cells, CHO cells, mouse cells and isolated DNA in solution. DNA irradiated in solution contains 30% more dimers than irradiated human cells growing on petri dishes. This difference is presumably due to the absorption of UV-light by the cell cytoplasm. We have found that human cells growing in a flattened configuration on plates, contain more UV-light induced dimers than rodent cells that grow in a more rounded fashion. CHO cells contain one-third fewer dimers than human cells after exposure to the same fluence of UV-light. Normal mouse cells contain the

same amount of cyclo-dimer as human cells; however, a transformed mouse cell line (RAG) contains one-half as many dimers as human cells. These data certainly indicate that in UV-light studies, the actual damage received by the genomic material of cells should be taken into account in the comparison of damage and repair in various biological systems.

#### **New Levels of Resolution in DNA Repair Measurements Made by Bromouracil Photolysis**

DNA excision repair can be assayed by a number of methods with varying degrees of specificity and sensitivity. In the bromouracil photolysis assay, bromodeoxyuridine (BrdUrd) is inserted into the repaired regions of the genome. Photolysis of these regions with 313 nm light yields information on the number and size of the average repaired patches in the DNA. Recent improvements in the method now enable us to measure DNA repair after very low doses of UV light well within the limits of doses incurred naturally. These improvements also permit us to measure repair at very short times after UV, during the more rapid phases of cyclobutane dimer and (6-4) pyrimidine pyrimidone removal.

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**CHROMOSOME CHEMISTRY**

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The major goal of this laboratory is to analyze and understand the macromolecular structure of eukaryotic chromosomes and their relation to DNA packaging, transcription, and replication. Our laboratory employs a wide range of biophysical, biochemical, and ultrastructural techniques to work towards detailed macromolecular models.

**Chromatin Structure in the Hypotrichous Ciliated Protozoa**

All ciliated protozoa exhibit nuclear dimorphism i.e., the existence of a transcriptionally-active macronucleus in the same cytoplasm with an inactive micronucleus. The hypotrichous ciliated protozoa possess two distinct nuclear features that distinguish them from other ciliates: (1) macronuclei consist of a "bag" of high polyploid (ca.  $10^4$ -fold), short (ca. 2-3 kbp), linear DNA molecules of low sequence complexity -- each fragment probably corresponding to an individual structural gene and its regulating flanking sequences; and (2) macronuclear DNA replication is localized exclusively in a Replication Band (RB) that migrates along the nucleus during S phase. Both of these features are unique in biological systems and offer considerable advantages in the study of nuclear structure and function compared to typical eukaryotic nuclei. During the past two years, our research has capitalized upon both of these nuclear features: (1) Macronuclear genomic libraries have been constructed, from which several genes have been cloned and sequenced, including the 5S RNA and polyubiquitin gene. In addition, nucleosome positioning studies of 5S RNA minichromosome have been completed; similar studies are underway with the polyubiquitin gene. (2) The replication band has received intensive cytochemical, ultrastructural and physiological study. An *in vitro* assay for DNA synthesis by isolated macronuclei was developed. Employing a macronuclear DNA expression library, DNA clones have been retrieved which code for replication band specific proteins. The DNA-specific osmium ammine stain was used to study the ultrastructure of replication bands. A surprising observation was the finding that replication bands have lectin-like properties, e.g., the binding of certain glycoproteins with high specificity. Current studies emphasize the identification of genes for DNA polymerases and associated proteins and the monitoring of their expression during the vegetative cell cycle.

**Three-Dimensional Reconstruction of Electron Microscope Tomography**

We have been interested in the 3-D reconstruction of asymmetric organelles, specifically of chromosomal structures during transcription, replication, and higher-order packaging. Most attention has been focused upon a chromosomal region of RNA synthesis, the Balbiani Rings (BR) of

Chironomus salivary gland cells. This gene is present on highly polytene chromosomes (ca.  $10^4$  endoreplicated), and, when active, generates a "puffed" region in the chromosome body. In the electron microscope, electron-dense nascent ribonucleoprotein granules (RNP) can be observed surrounding the chromatin axis. We have emphasized reconstruction of thick sections which cannot be visualized in a conventional, 100 KV, transmission electron microscope. To solve this problem, we have collected data on an intermediate voltage electron microscope (IVEM) as well as an energy filtering microscope (Zeiss 902). Both modes of data collection were shown to be useful and acceptable. In fact, since the method of energy filtration has only recently become commercially available, its use for imaging thick sections is not universally accepted. We undertook a collaborative study with a physicist, C. Colliex (Univ. Paris-Sud, Orsas, France), in order to demonstrate the theoretical and qualitative reliability of the method.

Another collaborative study with an inorganic chemist, Bruce Moyer (Chemistry Division, ORNL), has led to the development of an improved DNA-specific stain for the electron microscope. We plan to use this stain in studies to trace the 3-D path of DNA in various states of the cell nucleus.

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## Cancer Biology Section

### Section Overview - R. J. M. Fry

This Section is concerned with studies of (1) the mechanisms of carcinogenesis at the molecular, cellular, tissue, and whole-animal level, and (2) both the fundamental and the pragmatic aspects of risk estimates for radiation-induced cancer. Our studies in this latter area, a special responsibility of DOE, involve members of other sections and divisions and collaborative studies in other laboratories. The wide range of expertise and special radiation facilities required for some of these studies can only be found in the national laboratories.

The disbanding of the Toxicology program has resulted in the welcome return of A. Marchok and her colleagues to the Cancer Biology Section. This strengthens and extends the program of *in vivo-in vitro* studies of carcinogenesis in the tracheal epithelium. Many of the model systems currently used in various laboratories throughout the country that are studying carcinogenesis in the respiratory tract have been developed in this division. A recent example is the development of a variation of the tracheal implant which is an open-ended system, amenable to exposure studies with gases, liquids, particulates, or even chemical agents delivered in a pellet form.

People are not exposed to a single carcinogen, but the exposure to one type of carcinogen is usually at a higher level than any other of the many environmental agents. At low-exposure levels the probability that a single agent will cause cancer is small. The question is whether various agents act synergistically and to what degree. Some agents can be very weak carcinogens but act as potent promoters. As reported below, protracted exposure to formaldehyde can cause expression of the cancer potential of cells initiated with chemical carcinogens. The protracted nature of the exposures to promoters or co-carcinogens appears to be important. During the protracted period a number of changes, some of which may be sequential, must occur. This sequential change in gene expression remains to be characterized.

Tracheal cells in culture have been used by M. Terzaghi to study the mechanism by which normal cells appear to suppress the expression or the growth of neoplastic cells. Cell density and cell-to-cell contact are known to influence growth, but the mediators of the growth are not completely understood. There are two types of transforming growth factors TGF  $\alpha$  and  $\beta$ . The differences in production of TGF  $\beta$  and sensitivity to this factor between normal cell variants with enhanced growth and irradiated cells are complex. These studies have revealed another possible pitfall in the interpretation of *in vivo* processes from *in vitro* studies. The need for comparing results obtained *in vitro* is emphasized by the finding that instead of inhibition seen *in vitro*, TGF- $\beta$  induces cell proliferation in intact tissue.

We assume that the differences in the effects of high- and low-LET radiation are quantitative and not qualitative. However, some responses to the two types of radiation may differ at a mechanistic level. Whether the modulation of damage, not related to DNA repair, found after exposure to low-LET radiation also occurs after high-LET radiation must be established.

Differences in the cancer inducing effects of high- and low-LET radiation are an important field of research in the radiation carcinogenesis program. Risk estimates for neutrons and heavy ions will have to be derived from animal experiments because there are no human data. In order to make the estimates, studies at low doses of both neutrons and heavy ions are required. To make these risks derived from mice useful to human risks, methods of extrapolation from mouse to man are required. This year we report a possible method of extrapolating risks across species, which, if validated, could be used in conjunction with direct estimates of the effects of various high-LET radiation to establish appropriate risk estimates for humans. The acceptance of our proposed method of extrapolation would solve a number of pressing problems in radiation protection.

The study of growth factors, their receptors and the associated genes has become an important area of cancer research. The investigations of S. J. Kennel and his coworkers involve a cell surface protein with certain similarities to growth factor receptors. The protein, TSP-180, is encoded by normal cellular genes and the question arises as to whether this protein is a receptor for growth regulators. Certainly, the expression of the gene, as indicated by the levels of the specific protein, differs markedly in tumors of the lung and normal lung tissue. The power of two-site monoclonal antibody assays is shown by the quantitation of this surface protein. The correlation of metastatic potential of tumor cells and the amount of TSP-180 may reveal insights into our understanding of metastasis and invasion of tumor cells.

S. J. Kennel has developed a monoclonal antibody that binds to endothelial cells. The surprising finding is that the antibody appears to bind preferentially to endothelial cells of the lung. The possibility of developing antibodies to endothelial cells that are organ specific would have great potential for imaging and treatment.

Gene expression and regulation are germane to all cellular functions. The enzymes produced in liver provide convenient markers for many genes that are hormonally controlled. In order to unravel the complexities of interaction of agents that influence expression of a specific gene, the gene and its products must be characterized. F. T. Kenney and his coworkers have continued a meticulous dissection of a gene that is regulated by several hormones and have relied on diverse approaches to monitor the fluxes of the encoded messenger RNAs and proteins.

In parallel studies, F. T. Kenney and coworkers are attempting to identify a genomic regulatory element for insulin. These studies have

been facilitated through interactions with L.-Y. Chang, D. M. Yang, and W. K. Yang in the section and also with collaborators at the University of Virginia and NCI.

W. K. Yang and coworkers are concerned with changes in the regulation of gene expression induced by carcinogenic agents. An interesting aspect of the studies on murine leukemia virus (MuLV) and related proviruses is the organ specificity of the occurrence of RNA transcripts both in tissues that the related leukemias develop and in normal tissues.

It has been found that carbon tetrachloride results in the emergence of proviral DNA intermediates and greater levels of MuLV-related RNA transcripts. This observation opens a potential path of investigation of the action of co-carcinogenesis, mechanisms of which are poorly understood.

Other studies are concerned with the stability of the genome, the consequence of induction of instability, and the manner in which instability can lead to cancer. Initial findings that an endonuclease produced by murine leukemia viruses may cause a genomic instability that results in oncogenesis are intriguing.

#### INDUCTION AND PROGRESSION OF CARCINOGENESIS IN THE RESPIRATORY TRACT

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A clear understanding of how potential carcinogenic agents, including chemical and nuclear byproducts of energy production, may cause lung cancer requires analysis of several steps in the carcinogenic process. These steps include (a) initial interaction of one or more of the hazardous agents with macromolecular components of the target cells; (b) determination of whether the agent(s) is inducing cancer or promoting the response of initiated cells; and (c) identification of molecular and cellular changes that occur in the target cells during the multiple stages of carcinogenesis. To investigate many of these steps in respiratory tract carcinogenesis, we have developed *in vivo-in vitro* and *in vitro* rat tracheal cell models, and recent studies using these models are described.

#### Comparative *In Vivo-In Vitro* Studies of Carcinogenesis Induced by Split Doses of 7,12-Dimethylbenz(a)anthracene (DMBA), Benzo(a)pyrene (B(a)P) or Formaldehyde (HCHO)

*In vivo* models of tumorigenesis are essentially limited to morphological endpoints. To overcome this limitation a combined

*in vivo-in vitro* model of carcinogenesis, the tracheal implant was developed several years ago. In this model, rat tracheas are implanted on the backs of isogenic hosts to serve as a well-defined target organ for exposure. The sites of tumor initiation (TIS) are detected as carcinogen-altered cells selected by placing 14-day primary cultures established from pieces of the tracheal implants in a medium deprived of pyruvate, a component we found to be necessary for the long-term growth of normal tracheal epithelial cells but not altered cells. Our early studies demonstrated a direct correlation between extent of exposure to the carcinogen, DMBA, and number of TIS isolated from the tracheas as well as expression of the other markers in these cell populations. Also, since lesions on the explants are initially identified from cytopathology of exfoliated cells, cellular and biochemical properties of cell populations derived from the specific lesions were studied and were found to correlate directly with conventional morphological markers of the progression of neoplasia.

Recently, we modified the tracheal implant model into an open-ended system. In the open-ended tracheal implant (OETI) model, this well-defined target site can be exposed to unlimited numbers of exposures of single or multiple test agents of any physical form. During the past 2 years we have used this approach to study the relative potency of DMBA, B(a)P and HCHO to initiate carcinogenesis in the tracheas. OETI exposed twice weekly to 18.5  $\mu\text{g}$  DMBA-gelatin pellets for 2.25 months (333  $\mu\text{g}$  total) exhibited 4.5 TIS/OETI, while 76  $\mu\text{g}$  B(a)P-pellets given twice weekly for the same length of time (1368  $\mu\text{g}$  B(a)P total) induced only 0.50 TIS/OETI. Assuming linear relationships between carcinogen dose and induction of Tumor-Initiation Sites, DMBA is 36 times more potent than B(a)P. However, when 0.1% or 0.2% solutions of HCHO, a suspect carcinogen, were given twice weekly for 2.25 months, no TIS were detected. We questioned whether the HCHO had initiated carcinogenesis in some cells which had not reached the stage detected by our selection procedures. When we changed to the selection media at 63 days, 0.25-0.33 TIS/OETI could be detected in the HCHO-exposed trachea after 2.25 months of exposures. However, these were minimally altered cells which had not acquired immortality (not subculturable). We recently determined that exposing OETI to 0.1% HCHO for 1 year still did not induce TIS detectable after 14 days of culture. However, 3.7 TIS/OETI could be detected if the cell populations were allowed to replicate in culture for 63 days before selection. These findings support the view that HCHO, alone, is a definite, but very weak, carcinogen.

#### **Initiation-Promotion and Co-Carcinogenesis with B(a)P and HCHO**

Another advantage of the OETI is that, for the first time, tumor promotion studies can be carried out in the airway similarly to that classically done in skin tumor promotion. We completed a study in which tracheas were exposed to DMBA for 2 weeks followed by twice weekly exposure to TPA for 3 and 6 months. Control tracheas were exposed to DMBA or TPA, alone. TIS detectable in DMBA-exposed OETI by the 14-day primary cell culture selection procedure decreased from 2.5 to 1.4 between

3 and 6 months after exposure. Tracheas exposed to the DMBA followed by TPA for 3 or 6 months had 3 and 4 TIS/OETI, respectively, indicating that TPA not only promoted cells which were not permanently altered by the carcinogen, but may have promoted nondetectable initiated cells as well. TPA alone induced no TIS.

To determine whether HCHO would promote carcinogenesis initiated by B(a)P, we carried out a study in which OETI were first exposed twice weekly for 1 month to 170  $\mu\text{g}$  B(a)P-gelatin pellets followed by twice weekly exposure to 0.1% HCHO for 4.5 or 11.5 months. The B(a)P, alone, initiated only 0.33 TIS/OETI detected by 14 day selection, and an additional 0.67 were detectable when 63 day selection in culture was used. In OETI exposed to the B(a)P followed by HCHO for 4.5 months, 0.67 TIS/OETI were detected by 14 day selection, and additional 2.33 per trachea were detected by 63 day selection. After 11.5 months promotion, 3.33 TIS and 2.67 TIS per OETI were detected with 14 day and 63 day selection, respectively. These results show a fivefold increase in the early detectable (14 day selection) TIS above that obtained with B(a)P alone. Under these conditions no TIS are obtained by exposure to HCHO, alone. These results have far-reaching implications if extrapolated to the human situation, for they suggest that humans that have been exposed to polycyclic hydrocarbons may be far more susceptible to tumor development if exposed to HCHO. The findings are also consistent with the high incidence of bronchial carcinomas found in tobacco smokers, since tobacco smoke contains considerable amounts of both polycyclic hydrocarbons and HCHO.

We have also studied the induction of carcinogenesis when B(a)P and HCHO were delivered as co-carcinogens. OETI were exposed twice weekly for 4.5 months to 20  $\mu\text{g}$  B(a)P pellets followed 24 h later to 0.2% HCHO. Under these conditions, a high incidence (14 days plus 63 days selected) of 7.83 TIS were induced per OETI compared to 2.37 obtained with B(a)P alone or 0.25 obtained with HCHO alone. However, if HCHO was given 2.5 h before the B(a)P, an actual decrease to 1.49 TIS/OETI was found. Current experiments indicate that the inhibitory effect of HCHO is lost when the time between HCHO and B(a)P is increased to 20 h, and an actual enhanced effect currently estimated to be 4.5 TIS/OETI is expected.

#### **Interaction of Carcinogens at the Molecular Level and DNA Damage in Tracheal Cells**

To learn what role(s) interactions of carcinogens with macromolecules of the target cells play in enhancing or inhibiting the induction of carcinogenesis, we are investigating the induction of DNA-protein crosslinks (DPC) and single-strand breaks (SSB) in DNA from a nontumorigenic tracheal epithelial cell line, primary cell cultures, and in tracheal implants exposed to B(a)P and HCHO using alkaline elution techniques. We found that, as expected, HCHO induces a high level of DPC in both types of cell cultures, which were repaired within 16 h after exposure. B(a)P alone did not cause DPC; however, exposure to B(a)P 24 h before 90 min exposure to HCHO inhibited the number of DPC without

inhibiting the rate of repair. These results suggest that B(a)P-DNA adduct formation may be altering HCHO induced DPC. B(a)P or HCHO alone caused SSB, and exposure to both agents had an additive effect. An interesting finding was that a portion of the SSB induced by B(a)P was rapidly repaired while an increase in SSB appeared after 24 h that was not rapidly repaired. We proposed that the late appearing SSB are the result of excision of B(a)P adducts. Recently, considerable effort was expended toward modifying the alkaline elution technique to detect DPC in tracheal implants exposed to B(a)P and HCHO. Although repair of the extensive amount of HCHO-induced DPC found in the tracheal implants was slower than in culture, similar B(a)P-HCHO interactions were found. The induction of single- as well as double-strand breaks, the influence of HCHO on B(a)P adduct formation, and the correlation of these DNA modifications with carcinogenesis in the tracheas will be studied in future experiments.

#### **Growth Regulatory and Biochemical Changes in Carcinogen-Exposed Tracheal Cells *In Vivo* and *In Vitro***

A major interest in our laboratory is to identify key biochemical changes in carcinogen-exposed tracheal cells and to determine how they relate to the evolving changes in growth regulation found during the progression of neoplasia. Since we found that the ability of tracheal cells to survive in pyruvate-deprived medium is a very early marker of carcinogen-induced alterations, we have attempted to elucidate the cellular mechanisms underlying the requirement of pyruvate by normal tracheal cells in culture and the lack of this need by the carcinogen-altered cells. Two key earlier findings were: (1) normal tracheal cells actually metabolize [<sup>14</sup>C]pyruvate for macromolecular synthesis and lactic acid production at 3-4 times higher levels than carcinogen-altered cells; (2) carcinogen-altered cells and tumor cells have markedly higher levels of particulate-bound NADP<sup>+</sup>-dependent malic enzyme activity. This enzyme catalyzes the formation of pyruvate from malate. Recently, we have established conditions for culturing dissociated normal tracheal epithelial cells in a 1% serum-supplemented or a serum-free medium and have obtained preliminary evidence that the pyruvate is converted to one or more of its metabolites before taken up by these cells. NMR studies will be carried out in collaboration with the Analytical Chemistry Division to identify the metabolites and their differences in normal and carcinogen-altered cells from different stages of carcinogenesis. Experiments will also continue to characterize further the regulation of mitochondrial-bound malic enzyme during these same stages of carcinogenesis.

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#### RADIATION CARCINOGENESIS

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The research of the radiation carcinogenesis program is carried out at whole-animal, tissue and cellular levels. With the collaboration of R. J. Preston and co-workers of the Comparative and Cellular Genetics Section the studies have been extended to the chromosomal level, and with W. K. Yang to the molecular level.

## Effect of Cell Interaction on Neoplastic Development

We have investigated the effect of cell density on radiation-induced transformation in rat tracheal epithelial cells and the role of inhibitor(s) released by senescing normal epithelial cells. With increases in cell density from 10 to >100 colonies per culture dish, the frequency of enhanced growth variants (EGV) in X-irradiated cultures decreased from 1.5% to 0.9% and in control cultures from 1.3% to 0.09%. Thus, with increased cell density from 10 to 100 colonies per dish, the ratio of EGV frequencies in irradiated to control cultures of rat tracheal epithelial cells increased from 1 to roughly 10. The question now is, why does increased density have less effect on irradiated cells than on the control of cell populations? Further, experiments suggest that this effect of high density cultures is mediated via an inhibitor(s) released into the medium. Non-exposed control cultures appear to be more sensitive to inhibition by the inhibitor(s) present in conditioned medium than irradiated rat tracheal epithelial cell cultures. The inhibitor(s) is maximally detected in culture medium at a time when epithelial cells appear to be terminally differentiating and is not detected in the medium of proliferating normal rat tracheal epithelial cells. These results suggested that transforming growth factor type  $\beta$  (TGF- $\beta$ ) production and sensitivity to TGF- $\beta$  inhibition of growth played an important role during neoplastic development in irradiated rat tracheal epithelial cells in culture.

We have obtained data, using a receptor-binding-competition assay, which indicate that the inhibitor produced by senescing rat tracheal epithelial cells (see above) is transforming growth factor type  $\beta$ . TGF- $\beta$  has been reported to induce inhibition of cell growth and terminal differentiation in normal epithelial cell populations. TGF- $\beta$  is, in general, produced in a latent form and must be activated before it will bind to specific cell membrane receptors and inhibit cell growth. All neoplastic rat tracheal epithelial cells tested to date are insensitive to inhibition by TGF- $\beta$ , whereas all normal primary cultures are extremely sensitive. Preneoplastic cells appear to display intermediate sensitivity. Senescing normal cells were found to produce large amounts of TGF- $\beta$  in an active form. Proliferating preneoplastic and neoplastic rat tracheal epithelial cells either do not produce TGF- $\beta$  or produce it in a latent form. Our observations suggest that as a cell progresses from a normal to a neoplastic state both the response to active TGF- $\beta$  and the ability to produce active TGF- $\beta$  is lost.

We are currently investigating the possible relevance of these observations to the evolution of the neoplastic phenotype *in vivo*. Initial experiments have involved evaluation of the effect of TGF- $\beta$  on normal intact tissue maintained in perfusion chambers. Under these conditions TGF- $\beta$ , surprisingly, induced a marked cellular proliferation, in the absence of any apparent induction of cell differentiation. Therefore, the apparent importance of TGF- $\beta$  production in culture may reflect an artifact of the cell culture model. Further investigations are required to establish the possible relevance of development of resistance

to TGF- $\beta$  with neoplastic progression in culture to the effects of TGF- $\beta$  in intact tissue during neoplastic progression *in vivo*.

Marked differences between moderation of high LET (neutrons) and low LET (X rays) radiation-induced neoplastic transformation in the intact rat trachea have been observed. Ongoing experiments suggest that potentially oncogenic damage induced by exposure to low-LET radiation is rapidly repaired in the intact tissue. This phenomenon does not appear to be explained by a delay in division characteristic of the intact trachea. Cells irradiated in suspension and immediately seeded in culture do not divide for 24-48 h, and yet little repair of radiation damage occurs compared to actively dividing cultures irradiated 24-48 h after plating. After irradiation of the intact tissue with high-LET radiation, no repair (i.e., decrease in transformation frequency) is observed. The transformation frequency was similar to that observed in cells irradiated with high-LET radiation in suspension or in culture. These observations suggest that some attribute of the intact tissue, perhaps involving direct cell-cell contact within the intact tissue, is important in the modulation of damage associated with low-LET radiation.

#### Time and Dose-Response Relationship

The shapes of the dose-response curves for cancer induction are crucial for risk estimation and for modeling of the process of radiation carcinogenesis. A major role for experimental animal studies is the determination of time dose-relationships. Most of the human data is from high dose-rate exposures. In fact, estimates of the influence of lowering dose rate have not been obtained from human studies, and it is unlikely that they will be. Also, there have been no systematic fractionation studies. We know the effect of fractionation for one or, at the most, a small number of fractionation regimens on a small number of tissues. One current model of dose-response relationship for radiation-induction of cancer depends on the linear-quadratic response. The current views about the influence of spatial and temporal relationships, suggest that the initial slope of the linear-quadratic response will be the same irrespective of whether the exposure is single and at a high dose rate, or in multiple small fractions, or at a very low dose-rate. This hypothesis has been tested for the induction of lung and mammary tumors in BALB/c mice. The tumors have very different dose-response relationships. In the case of lung tumors the apparent linear component predominates over considerable dose range about 0-0.45 Gy compared to about 0.05 Gy in the case of mammary tumors. In both tumor types, reduction of the dose rate reduced the effectiveness of tumor induction. In the case of lung tumors, the high dose-rate response (0.35 Gy/min) could be described by the regression,  $y = 11.8 + 0.041X + 0.00043X^2$  and at 0.083 Gy/day,  $y = 12.5 + 0.043X$ . In the case of fractionation regimens when the same total dose (2.0 Gy) was given in 0.1 Gy fractions, the tumor incidence was reduced to that obtained with the low-dose rate exposures. Fractions of 0-5 Gy had a greater effect than 1.0 Gy but less than the single high dose-rate exposure of 2.0 Gy. These results are consistent with a model that

predicts the effect at very low doses or dose rates becomes dose rate independent and total dose dependent.

The use of single risk estimates for cancer mortality or incidence for protection standards without weighting for protraction and fractionation is becoming increasingly inappropriate. The information on which to base these weighting factors must come from animal experiments.

### **Skin Carcinogenesis**

The complexity of the effects of fractionation and the lack of understanding of the mechanisms involved are underlined by our findings with fractionation and the induction of skin cancer by soft X rays. We have previously reported that exposures of 250R 25 kV X rays twice a week for 2, 4 or 8 weeks were ineffective in producing frank skin cancers but that 8 or 16 exposures initiated many cells with the potential for cancer development. The expression of the initiated cells and the development of cancers could be brought about by post X-irradiation exposures to 12-O-tetradecanoylphorbol-13 acetate (TPA), ultraviolet radiation (UVR) or psoralen-plus UVA (PUVA). Now we have found that with the same total dose, which given in 16 fractions produces about 1% of skin cancers, results in an incidence of about 90% if the dose is split into 64 fractions. Clearly, most of the large number of exposures are influencing expression and not initiation. These results, together with those with UVR, PUVA and TPA, suggest a remarkable lack of specificity in "promoting" capability. Furthermore, X rays are as potent a promoter as TPA in the skin. The skin model provides an opportunity of using *in situ* hybridization to study sequentially the changes in gene expression, especially those associated with growth factors (collaboration with W. K. Yang).

### **Experimental Animal Data and Human Risk Estimates**

The new dosimetry for the atomic bomb survivors has dashed the hopes of deriving risks for cancer induction in humans by neutrons. Such risk estimates must now come from experimental animal data. Two types of information must be available. First, the initial slopes for the neutron dose-response curves for cancer induction from the appropriate organs must be obtained. Second, an acceptable method of extrapolation of the radiation risk estimates from animal data to human risks must be found. The first of these studies is in progress.

We have recently reported the results of a series of experiments and a method of extrapolation of risk estimates across strains of mice and from mouse to humans. The underlying questions were (1) whether the relative risk or the absolute risk was the model of choice for various solid cancers and leukemias, and (2) whether the natural incidence influenced the susceptibility to induction of cancers. It was found that in general susceptibility is determined by natural incidence. The relative risk model was found to be the more appropriate model for solid cancers. Neither model provided good fits for thymic lymphoma or

reticulum cell sarcoma, but both models appeared to fit the data for myeloid leukemia. Based on risks estimated for exposures to 1 Gy, remarkable concordance between humans and mice for cancer of the lung, breast and leukemia was observed. For example, for breast cancer in mice the relative risk was 2.0 with 95% confidence limits of 1.62-2.6 and in the atomic bomb survivors 1.9 with 95% confidence limits of 1.4-2.5.

These results are an encouraging beginning. Such questions as the importance of the age distribution in the mouse and human populations that we have compared must be tackled. While these recent studies involved data from about 9,000 mice of four different strains, data for different tumors and strains should be obtained.

The collaborative study with R. D. Ley, Lovelace Foundation, has resulted in the initial description of a tumorigenesis that is quite unusual. Exposure of the eyes of *Monodelphis domestica* to UVR causes damage to the corneal epithelium with subsequent hyperplasia. Next is seen neovascularization of the corneal stroma followed by de-differentiation and proliferation of the stromal fibroblasts. The vascularization increases and angiomatous lesions develop and may proceed to angiosarcomas in some animals. The most frequent type of tumor to develop is fibrosarcoma. Exposure to photoreactivating light after each exposure to UVR results in a marked reduction in tumors. This suggests that the induction of cyclobutane pyrimidine dimers is involved in the initial events of the induction by UVR of these tumors.

The sequential changes found in the development of the tumors of corneal stroma are different from any other reported tumorigenesis, because angiogenesis, a relatively late change in most tumors, is a very early change. The development of these eye tumors appears to be dependent on marked changes in the production of growth factors that must include an angiogenic factor and a fibroblast growth factor. The number and nature of pertinent growth factors remain to be determined.

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#### MONOCLONAL ANTIBODIES FOR DIAGNOSIS AND THERAPY

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#### Basic Carcinogenesis

Cell surface proteins mediate interaction between cells and their environment. In addition to uptake of nutrients, cells receive specific stimuli from growth factor-receptor interaction at the cell surface. Growth factors affect specific cell types in different ways, and it is now clear that aberrations of the normal factor-receptor function can lead to malignancy. Studies on growth factors and receptors identified as proteins analogous to oncogene products are ongoing in numerous laboratories. We have been examining a protein which also displays several characteristics of growth factor receptors, but differs from those described to date.

A tumor surface protein of 180,000 Mr (TSP-180) has been identified on cells of several lung and mammary carcinomas of BALB/c mice. TSP-180

was not detected on normal lung tissue, embryonic tissue, or reticulum cell or other sarcomas, but it was found on lung carcinomas and a melanoma from other strains of mice. Considerable amino acid sequence homology exists among TSP-180s from several cell sources, indicating that TSP-180 synthesis is directed by normal cellular gene(s) although it is not expressed at high levels in normal cells. The regulation of synthesis of TSP-180 and its relationship to normal cell surface proteins are being studied with the hypothesis that it may represent a receptor molecule for growth regulation factors.

TSP-180-like molecules have been identified on four different human carcinomas (lung, breast, embryonal, and colon). Evidence indicates that the human TSP-180 complex is analogous to, but distinct from, that on mouse cells. Two-site monoclonal antibody (MoAb) assays for TSP-180 in the mouse and the human have been developed which allow quantitation of low levels (ng/mg protein) of TSP-180 in normal and neoplastic tissue. Results in the mouse indicate that tumors have 10-100 times more TSP-180 than do normal tissues. In normal tissues, trace amounts of TSP-180 can be detected in lung and leg muscle, but not in heart, liver, kidney or spleen. Benign adenomas have small amounts of TSP-180 and the amounts increase as the adenomas become larger or progress to adenocarcinomas. All murine lung carcinomas tested to date, including primary, spontaneous, or chemically induced, from different strains of mice, contain high levels of TSP-180.

Similarly, induced and spontaneous mammary carcinomas express large amounts of TSP-180. Correlation of TSP-180 concentration with the malignant potential of the cells in this system is difficult to establish because of the multiple phenotypic changes that cultured mammary cells undergo. TSP-180 concentration correlates with the metastatic potential of Lewis lung carcinoma. Lewis lung cell lines that are capable of forming many spontaneous or artificial metastases have high TSP-180 levels, while variant cell lines of low metastatic potential have lower TSP-180 levels. A similar observation has been made for variant lines of B16 melanomas.

Analyses of human tissue show that TSP-180 is expressed at low levels in nearly all normal organs but is elevated in some carcinomas, i.e., colon, lung, and larynx. In contrast, no detectable levels of TSP-180 were found in human breast carcinomas.

Molecular characterization of TSP-180 has revealed five distinct forms. All forms are glycosylated, but only the largest is phosphorylated. Differential labeling experiments indicate that the larger forms ( $M_r = 204$  kDa and  $M_r = 185$  kDa) are external, while a membrane-bound, internal pool of smaller molecules ( $M_r = 150$  kDa,  $M_r = 135$  kDa and  $M_r = 116$  kDa) represents the majority of cell-associated forms detected by the two-site assay.

Monoclonal antibodies to TSP-180 have been used in immunoaffinity chromatography to isolate TSP-180 from tumor cell sources. These purified

proteins have been separated on SDS-PAGE and transferred to PVDF membranes for peptide mapping and amino acid sequencing. Data show strong analogy among bands 1-4 but that band 5 is different. N-terminal amino acid sequences of mouse and human band 5 are identical in seven out of eight positions.

Current work is focused on molecular characterization of TSP-180 and attempts to identify a specific receptor function for the complex. Molecular characterization is being approached by molecular cloning. A cDNA library of Line 1 cells in  $\lambda$ gt11 has been screened with a rabbit antiserum to TSP-180 and a consensus oligonucleotide from the N-terminal sequence of band 5. Several phage isolates are being characterized which appear to contain fragments of TSP-180 specific DNA. This DNA will be used to analyze mRNA sizes and synthesis as well as the gene's size and position in normal and neoplastic cells. The effects of known factors on the phosphorylation of TSP-180 and growth of Line 1 cells are being analyzed to establish a function for the TSP-180 complex.

MoAb to normal mouse lung cells have been developed to study the role of normal cells as precursors of different types of lung cancer and to probe the interaction among cells during metastasis to the lung. Rat MoAb to mouse lung macrophages, Type I cells and endothelial cells have been identified and characterized. The most remarkable of these are two antibodies that recognize different epitopes on a 112-kDa glycoprotein expressed exclusively on lung endothelial cells. This is the first demonstration that endothelial cells in the lung are different from those in other organs. The MoAb may be useful for organ specific drug delivery, for studying the interaction between tumor cells and endothelial cells, and for "negative imaging" of tumors for diagnosis.

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#### REGULATION OF GENE EXPRESSION

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The objectives of this research are to define in molecular terms the mechanisms controlling expression of specific genes in mammalian cells, how these mechanisms operate during differentiation, and how they are regulated by hormones and other specific effectors. Current focus is on a gene we have cloned and characterized that is transcriptionally enhanced by glucocorticoids and by each of the usually antagonistic hormonal agents, insulin and cyclic AMP. The response of this gene to insulin in cultured hepatoma cells is especially large and rapid, providing a model for analysis of the mechanisms by which interaction of the hormone with its membrane-bound receptor is realized as enhanced transcription of a specific nuclear gene.

#### Structure of Gene 33

We have recently completed characterization of the structure of this multihormonally-regulated rat gene, provisionally termed gene 33 pending identification of the protein product. The structural gene spans 13.5 kbp of DNA, of which only 2.97 kb are represented in the mature transcript. Coding sequences are present in four exons, the last (3'-terminal) containing 85% of the total. In the 5'-flanking DNA there are TATA and CAAT boxes in their usual positions (-31 and -89, respectively), as well as three GC boxes constituting a binding site for the Spl transcription factor and located between the TATA and CAAT sequences.

Such GC elements are generally associated with constitutive genes, while TATA and CAAT boxes are found in regulated genes; that the gene 33 promoter region contains both types of control elements is consistent with its being hormonally and developmentally regulated but expressed in all tissues examined.

### Gene 33 Products

To aid in characterization of gene structure and as a step toward identifying the protein product and its physiological function, we analyzed 14 clones carrying gene 33 cDNA sequences. These proved to be of two types, as two of the clones lacked internal restriction sites present in the other 12, but in both types restriction maps of the 5'- and 3'-regions were identical. The larger type of cDNA represents a transcript of 2970 nucleotides, and contains an open reading frame of 1377 nucleotides encoding a protein of 459 amino acids, molecular mass 49,919 daltons. This is in excellent agreement with our earlier estimate of the size of the protein synthesized in reticulocyte lysates from hybrid-selected mRNA (Lee et al., *J. Biol. Chem.* 260, 16433-16438, 1985).

Sequencing of the smaller type of cDNA reveals that it represents a truncated mRNA product derived from an uncommon form of alternative transcript splicing. Nucleotides 469 to 687, representing the 5'-end of the fourth exon in the larger transcript, are deleted from the smaller one. The deletion is within the protein coding region but the reading frame is maintained; the smaller mRNA potentially encodes a protein of about 42,000 daltons. That two mRNA products of this single copy gene are actually present in liver poly(A)-RNAs was established using an S1 nuclease protection assay; the smaller mRNA represents only 5 to 10% of the total. Thus in liver two discrete mRNAs are expressed; that both are found in polysomes is a strong indication that two protein products of this gene are synthesized as well. We are currently examining the possibility of differential expression of these gene products in various tissues and in response to the inducing hormones.

The deduced amino acid sequences of the protein products were entered into PIR and Genbank databanks and proved to have little resemblance to the sequences stored therein. Various sequence properties suggest a soluble, intracellular protein. A search of the sequences for functional domains was not informative.

The intact cDNA, and a version from which the GC-rich untranslated portion was removed, were cloned into *E. coli* expression vectors in attempts to produce the protein in sufficient quantity for biochemical characterization and antibody production. As yet a significant degree of expression has not been achieved. We are also exploring synthesis of a selected oligopeptide with presumed immunogenic properties to be used for production of specific antibody and, thereby, isolation and characterization of the protein products.

## Hormonal Regulation

We have used several approaches toward defining sequence elements in the DNA within or flanking gene 33 that may be involved in its transcriptional enhancement by hormones, with emphasis on insulin as an almost totally undefined regulator of gene expression. DNase I-hypersensitivity and gel retardation assays have not been consistently successful but are continuing.

Recombinant plasmids were constructed in which gene 33 5'-flanking DNA sequences were placed upstream of the *E. coli* chloramphenicol acetyltransferase (CAT) gene; these were transfected into cultured cells via CaPO<sub>4</sub> precipitation and the transcriptional promoting capacity of the gene 33 DNA determined as transient expression of CAT activity. Constructs containing 0.5 or 2.6 kbp of this rat DNA were very effective in driving CAT expression, and this expression is significantly enhanced by insulin. However, we have not yet been able to match the tenfold or greater increase in expression of the endogenous gene in these cells.

A third approach toward identifying an insulin regulatory element is a collaborative effort with J. Larner (University of Virginia) and A. Larner (NCI), who used the technique of transcription from added DNAs in nuclear extracts. Using our gene 33 DNA containing 500 bp of transcribed sequences and 1500 bp of 5'-flanking elements, they also found effective promoter activity that was doubled by insulin treatment prior to preparation of nuclear extracts.

The results of these experimental approaches are encouraging but not yet definitive. Extensions are currently being examined, including the preparation of new constructs containing 5'-flanking DNA further upstream as well as elements from within the gene, the latter with emphasis on the unusually large first intron. We are also deriving stable transformants of several cell lines. Conceivably entirely novel approaches may have to be devised for the elucidation of mechanisms involved in gene regulation by the most pleiotropic of all hormones, insulin.

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## MOLECULAR GENETICS OF CANCER

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The purpose of this research work is to elucidate the molecular genetic mechanisms of carcinogenesis, with particular emphasis on the impact of environmental carcinogens on genomic stability and regulation of gene expression in the cell. Our working hypothesis is that mobile gene elements in the cell genome play important roles in these two aspects of genetic control. Laboratory mice have been employed as an experimental model. Most of our recent research progress has entailed molecular cloning, structural characterization, and functional assessment of retrotransposable genes present in germ lines of the mouse.

Two particular families of endogenous retrovirus-like gene in the mouse genome have become better understood by our efforts. One is the murine leukemia virus (MuLV)-related proviral family, and the other is the MRL (the "MboI repeat"-containing LTR) proviral family, co-discovered by us.

Our previous findings established that the 30 to 50 individual copies of MuLV-related proviral genes constitute a distinct family. The characteristics of these genes are (1) that they utilize glutamine tRNA instead of proline tRNA as the primer for initiating the reverse transcription, (2) that they possess the Fv-1 B-tropism determinant in the gag-p30 gene region and the mink cell focus-forming (MCF) sequence specificities in the env gene region, and (3) that their LTRs contain a distinct 190-base pair (bp) to 200-bp "inserted" segment which is not detected in the proviral LTR of murine leukemia viruses. The MRL family comprises 100-200 copies of proviral structures and 3000-5000 copies of solitary LTRs per haploid mouse genome. Their biological significance is suggested by our previous works showing localization of some of its members on the mouse Y chromosome (i.e., male-specific) and also detecting expression of MRL-specific mRNA predominantly in the testes and ovaries of adult mice.

Recent findings concerning these two endogenous proviral gene families can be described briefly as follows: (a) The MuLV-related proviral gene family can be divided into two classes, polytropic and modified polytropic classes, as defined by Stoye and Coffin. We have found that these two classes can be distinguished by the LTR size (700-bp versus 750-bp), as well as by characteristic restriction enzyme sites within the structural genes. In the structural characterization, we also observed that specific sequence deletions are present in some proviruses of the two classes and that the deletions generally occur from sequences flanked by 7-bp direct repeats in the structural genes. (b) In normal

mice, MuLV-related RNA transcripts are detected mainly in the liver and kidney, indicating a tissue-specific expression distinct from murine leukemia proviruses which are expressed predominantly in lymphoid and hematopoietic organs. (c) Significant elevation of the MuLV-related transcript level occurs in the mouse liver after damage and subsequent regeneration induced by carbon tetrachloride (CCl<sub>4</sub>), a known co-carcinogen. More significantly, transient emergence of proviral DNA intermediates in the CCl<sub>4</sub>-treated mouse liver was also disclosed suggesting that retrotransposition of MuLV-related genes may be induced by this co-carcinogenic agent. (d) LTRs of MuLV-related proviruses showed very poor activities in expressing bacterial chloramphenicol acetyl transferase (CAT)-gene in mouse cells in an *in vitro* transcription assay. Our experiments involving dissection of the LTR structure demonstrated the presence of an apparent negative "enhancer" element in the 5' end region of the MuLV-related LTR for the CAT expression in the mouse cell. (e) A retrovirus vector system suitable for studying endogenous retroviral LTR elements has been established. This involves development of retrovirus-packaging cells of non-mouse origin (e.g., CCL64 cells) and molecular construction of recombinant DNA clones carrying different LTRs, packaging signal sequences and an oncogene (or a reporter gene). Oncogenes used successfully in this retrovirus vector system are *Ki-ras*, *Ha-ras*, *raf* and *fos*. (f) Preliminary results indicate that the endonuclease or "integrase" gene of murine leukemia viruses is potentially oncogenic, presumably by causing genomic instability in mouse cells. (g) We have successfully isolated the male-specific MRL-related proviral genes from a mouse genomic library. Sequence analysis revealed that the major type-specific sequence of this proviral structure is localized in the 3' region of "pol" gene and that this sequence comprises of a repetitive motif suggestive of a regulatory function. The sequence was subcloned and called the "A2." The LTR of this proviral sequence characteristically lacks an IS element that is found in other MRL genes and the LTR of MuLV-related provirus.

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## Molecular and Cellular Sciences Section

### Section Overview - J. S. Cook

The various projects in this Section are directed toward understanding at the most basic level the mechanisms of life processes that relate to the overall mission of the Biology Division. Programs are concerned with the structure of DNA and its organization in the eukaryotic genome, the structure of chromatin, DNA-repair processes, and enzyme mechanisms, all problems underlying cellular functions and the genetic responses of organisms to environmental factors. Other programs of the Section, also relating organisms to their environment, are concerned with the mechanisms of transport of metabolites by kidney cells and the responses of cells to freezing. The latter program also has an applied component in the preservation of valuable mutants. Some of the significant findings of the various groups are encapsulated here to demonstrate how the ongoing work is not only of interest as basic science but also relates to the programs of the DOE.

Together the Protein Engineering Group and the Protein Chemistry Group form the largest unified cohort in the Section. They use the methods of site-directed mutagenesis together with other sophisticated biochemical and genetic techniques to explore structure/function relationships of several proteins of both plant and animal origin. Although the proteins form a diverse group, they are each of significance to various aspects of the mission of the Biology Division and include energy-related enzymes, growth factors important to cancer biology, and repair enzymes that function in the responses of cells to physical and chemical mutagenic agents.

One such project is concerned with the REV1 gene of yeast, its gene product being involved in "error prone" repair that is the basis of mutagenesis in this organism by ultraviolet light and many chemicals. The gene has been cloned and the modifications of its cellular functions are being studied in transformants that have incorporated specifically engineered versions of the gene. DNA repair and mutagenesis are also the focus of a group investigating repair of alkylated DNA by human repair enzymes, correlating chemical and genetic effects by the use of shuttle vectors between *E. coli* and cultured cells.

The group studying the Structure and Organization of a Eukaryotic Genome have extended their fine analysis of the relation of DNA primary sequences in their model system (a complex satellite of a crustacean) to mutation hotspots, showing that regions of sequence-dependent distortion of the helix are regions of high frequency mutation. The satellite sequence is transcribed in specific organs at specific growth stages, and the regulation of such transcription is under current investigation.

The group investigating the structural biology of nucleosomes is analyzing in detail their solution of structure at the 8 Å level and is

planning for crystals that will allow a 3 Å solution. This is one of the few groups in the world to have succeeded in crystallizing nucleosomes. The DNA wrapping around the protein core of the nucleosome does not form a perfect circle but at intervals makes sharp bends, related to the primary sequence at those points, which are putative binding sites for DNA binding proteins. The analysis has the promise of establishing the structural basis for the regulation of transcription and other DNA-dependent functions of the cell.

Studies on the regulation of messenger RNA have focused on three enzymes, a polymerase that functions in RNA synthesis and that is activated by phosphorylation of its terminal repeating sequence, a decapping enzyme that removes the 3' terminal nucleotide of mRNA and thereby renders it susceptible to degradation, and a 3' → 5' exoribonuclease that degrades decapped RNA. The interactions of the activities of these enzymes appear to play important roles in regulating mRNA concentration and translation.

The Membrane Biology group has continued its studies on the regulation of transport systems in relation to cell growth and differentiation. Growing cells, transformed cells, and cells responding to tumor promoters have a higher capacity for uptake of essential metabolites than quiescent or differentiated cells. A recent significant finding from this group is that the higher level of amino acid transport in growing and/or promoted cells is supported by the protein kinase C-mediated phosphorylation of a regulatory component of the transport system.

The Cryobiology group has supported its contention that an important but hitherto neglected component of freezing injury is the crowding of cells in unfrozen channels during ice formation. The combination of osmotic shrinkage, cold, and crowding under pressure (centrifugation), are damaging even without ice formation, as their theory proposes. They have also continued applied studies in freezing of embryonic mouse and *Drosophila* for the cost-efficient, long-term banking of mutant stocks.

Several members of the Section are collaborating with scientists in other divisions on problems in Structural Biology. These projects (and the other divisions concerned), include studies on viruses and other macromolecules by scanning tunneling microscopy (Health and Safety Research Division), sequencing by Fourier transform mass spectrometry of mutagenized DNA fragments, including the specific identification of alkylated isomers of altered bases (Analytical Chemistry Division), structural studies on cyclodextrins to explore hydrogen bonding by neutron scattering, and refinement of the nucleosome studies described above (Solid State Division). These projects form the nucleus of a new Structural Biology Program.

**PROTEIN ENGINEERING AND PROTEIN CHEMISTRY**

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The unifying theme in this group is the use of site-directed mutagenesis and chemical approaches to elucidate structure/function correlations of proteins. Investigations focus on four proteins with quite diverse functions: ribulose biphosphate carboxylase/oxygenase, an enzyme whose activity is a major determinant of biomass yield; phosphoribulokinase, a photosynthetic enzyme whose activity is regulated by light; epidermal growth factor, a hormone that regulates cellular growth and differentiation, and DNA-*O*<sup>6</sup>-methylguanine methyltransferase, a protein that repairs alkylated DNA.

**Ribulose Biphosphate Carboxylase/Oxygenase (Rubisco)**

Ubiquitous among photosynthetic organisms, Rubisco is essential for net conversion of atmospheric CO<sub>2</sub> into carbohydrates. Thus, this enzyme is a major cornerstone of living processes and is highly relevant to the production of biomass for energy and to the global CO<sub>2</sub> issue (i.e., the greenhouse phenomenon). The enzyme is bifunctional: In addition to catalyzing the carboxylation of *D*-ribulose-1,5-biphosphate to yield two molar equivalents of *D*-3-phosphoglycerate (the CO<sub>2</sub>-fixation reaction), it also catalyzes the oxidation of ribulose biphosphate by molecular oxygen to yield one molar equivalent each of phosphoglycolate and 3-phosphoglycerate. Although multiple substrate specificities among enzymes are not unusual, the bifunctionality of Rubisco is perhaps unprecedented in that the two reactions catalyzed are the initial steps in competing metabolic pathways -- photosynthetic assimilation of CO<sub>2</sub> and photo-respiration, the latter an energy-wasteful process which results in the release of previously fixed CO<sub>2</sub>.

Our goals are (1) to understand the mechanism of this complex enzyme, especially the precise catalytic roles of active-site residues, and (2) to evaluate the feasibility of improving the carboxylase/oxygenase activity ratio and thereby providing an approach to enhancing biomass yields.

In contrast to Rubisco from higher plants, which is comprised of two gene products (eight large and eight small subunits per molecule of enzyme), the functionally analogous enzyme from the purple, non-sulfur photosynthetic bacterium *Rhodospirillum rubrum* is a homodimer and the product of a single gene. This enzyme has thus been used for all of our

mutagenesis studies, whereas chemical modifications have involved both types of the enzyme.

Affinity labeling, chemical cross-linking, and comparative sequence analyses provided strong, but indirect, evidence for the catalytic functionality of Lys-166 and Lys-329 and suggested an active-site location for Glu-48. Site-directed mutagenesis has confirmed a catalytic involvement for all three residues. Amino acid substitutions at each of these three positions abolish overall carboxylase activity but prevent neither the obligatory activation step (carbamylation of Lys-191 by  $\text{CO}_2$ ) nor substrate binding. Furthermore, the position-329 mutant proteins retain the ability to catalyze enolization of ribulose biphosphate (the first step in overall catalysis) so that the catalytic intervention by Lys-329 must be at some later step. In contrast, the position-166 mutant proteins are unable to promote enolization of substrate but retain the ability to catalyze conversion of a 6-carbon reaction intermediate to 3-phosphoglycerate (collaboration with G. H. Lorimer of DuPont), thereby establishing Lys-166 as an essential base in the enolization step.

To introduce subtle changes in the active-site microenvironment, we have converted Lys-166, Lys-191, or Lys-329 to aminoethylcysteinyl residues by a combination of mutagenesis and subsequent chemical modification. The net structural change is replacement of a lysyl  $\gamma$ -methylene group by a sulfur atom. Each of the three cysteinyl mutant proteins is catalytically inactive, but partial activity is restored by alkylation with bromoethylamine or ethylene imine.

We have also addressed a fundamental structural question by site-directed mutagenesis: Does each catalytic subunit of Rubisco contain an independent, functional active site or is the active site generated by intersubunit interactions? Both the Glu-48  $\rightarrow$  Gln and the Lys-166  $\rightarrow$  Gly mutant proteins are devoid of carboxylase activity; however, when the genes encoding these proteins are coexpressed in *E. coli*, an enzymically active hybrid is formed. This observation demonstrates that the active site is formed by interacting domains on separate subunits and that amino acid residues from both are necessary for catalysis.

### Phosphoribulokinase (PRK)

PRK catalyses the ATP-dependent synthesis of ribulose biphosphate in the illuminated chloroplast stroma. The enzyme is activated by thioredoxin-mediated reduction of a disulfide. Cys-16, previously identified by us as an active-site residue, was proposed as one of the regulatory sulfhydryls. This has now been demonstrated directly by sequencing of the tryptic peptides containing the four cysteinyl residues of PRK in conjunction with comparisons of selective sulfhydryl labeling patterns in the tryptic maps of oxidized and reduced PRK. The disulfide was shown to be intrasubunit and comprised of Cys-16 and Cys-55. The dual role of Cys-16 as an active-site residue and regulatory sulfhydryl suggested that inactivation might result from blockage of a catalytic group. However, complete methylation of Cys-16 resulted in only a 50%

loss of activity. The  $K_m$  for ATP increased sixfold while the  $K_m$  for Ru5P was unchanged, consistent with our earlier postulate that Cys-16 is located in the nucleotide binding domain. These results prove that Cys-16 is not required for catalysis and that blockage of this sulfhydryl alone does not explain oxidative deactivation. Disulfide-induced steric or conformational changes must be invoked to explain the complete loss of activity concomitant with oxidation. Changes in the intrinsic fluorescence of PRK upon oxidation are consistent with the latter possibility.

As a prerequisite to site-directed mutagenesis of spinach PRK, its gene has been cloned and completely sequenced. In addition to the sequence for the complete mature protein, cDNA clones of PRK encode a transit peptide at their amino terminal end. This is consistent with a nuclear location for the PRK gene and transport of the protein into the chloroplast following its synthesis in the cytoplasm.

### Epidermal Growth Factor (EGF)

Because of their crucial role in the regulation of growth and differentiation and the possible abnormalities caused by their malfunction, there is considerable interest in the study of growth factors. Among the most highly studied growth factors is EGF, a 6-kDa protein (53 amino acids) with 3 internal disulfide bonds. EGF initiates its action by high-affinity binding to its specific receptor on the cell surface -- the EGF receptor. The receptor is a single polypeptide having an internal domain exhibiting protein tyrosine kinase activity under the regulatory control of an external domain which binds the growth factor.

The binding of EGF to its receptor unleashes a cascade of biochemical events including autophosphorylation on tyrosine residues besides tyrosine phosphorylation of endogenous cellular proteins by the EGF receptor kinase, increased glycolysis and protein synthesis, and increased transcription of specific genes, leading ultimately to stimulation of DNA replication and cell division. Evaluation of the biological properties of the receptor has shown that the receptor tyrosine kinase activity is essential for most of the events that lead ultimately to cell division.

Although the EGF receptor had been cloned and many of its physical and catalytic properties examined by site-directed mutagenesis, little had been described about EGF's structure-function relationships. Our goals are (1) to identify the amino acid residues in EGF that are crucial for its binding to its receptor, (2) identification of residues critical for stimulation of the receptor's tyrosine kinase activity, and (3) development of EGF analogs that could possibly act as antigrowth substances.

A synthetic chimeric gene, coding for human EGF fused to the signal peptide of *E. coli* alkaline phosphatase, was cloned into *E. coli* under the transcriptional control of the *tac* promoter. Induction with isopropylthiogalactoside led to the secretion of the correctly processed

EGF into the bacterial periplasmic space. The protein was purified to homogeneity and found to be identical to authentic human EGF in its structure, sequence, receptor binding and stimulation of receptor tyrosine kinase activity. Structure-function analysis was initiated with alterations of targeted amino acid residues by oligonucleotide-directed mutagenesis.

The receptor binding affinity of each mutant, relative to the wild type, was measured by both radioligand competition and receptor tyrosine kinase stimulation assays. In general, the values obtained by the two methods were in agreement for each EGF species and followed the order: wild type > Glu-24→Gly > Asp-27→Gly > Pro-7→Thr > Met-21→Thr > Tyr-29→Gly > Tyr-22→Asp > Leu-47→His > Ile-23→Thr > Tyr-37→Gly > Arg-41→Gly. The relatively low values obtained with substitutions of Tyr-22, Ile-23, Tyr-29, Tyr-37, Arg-41, or Leu-47 point out the importance of these residues in the biological activity of human EGF. From the kinetics of the kinase assays it was apparent that more than one EGF molecule interact cooperatively in stimulating activity.

With the exception of the Ile-23→Thr and Leu-47→His mutants, the different EGF species retained the ability to stimulate the receptor's tyrosine kinase activity close to that of wild type. The Ile-23→Thr and Leu-47→His mutants stimulated the receptor to maximal rates of only 16% and 30%, respectively, of wild type EGF. These mutants were also able to displace wild type EGF and partially reduce the receptor's tyrosine kinase activity *in vitro*. Studies are in progress to test these two analogs as possible inhibitors of cell proliferation.

#### DNA- $O^6$ -Methylguanine Methyltransferase

The *ada* gene of *E. coli* plays a central and unusual role in repair of alkylation damage in DNA. The 39-kDa Ada protein (DNA- $O^6$ -methylguanine methyltransferase) acts both in repair and in its own regulation. This and other similar methyltransferases react uniquely in a stoichiometric and irreversible fashion by accepting alkyl groups from specific adducts in DNA. The globular, monomeric Ada protein has two functional domains each containing a specific alkyl-acceptor cysteine residue. Cys-69 in the N-terminal domain is specific for methyl phosphotriesters in DNA, while Cys-321 in the C-terminal domain is an alkyl acceptor for  $O^6$ -alkylguanine (and  $O^4$ -alkylthymine) in DNA. The Ada protein methylated at Cys-69 is a transcription activator of its own and other genes of the *ada* regulon. The structure of the Ada protein is being probed by oligonucleotide-mediated site-specific mutation of its cloned gene. In prerequisite experiments, this approach was used to construct a plasmid with a high level expression of the wild type Ada protein which is being used for its physical studies. Subsequently, two mutant Ada proteins were created, both of which have Cys-321 replaced by another nucleophilic amino acid, histidine. A study of the pleiotropic functions of these mutant proteins showed not only their loss of  $O^6$ -methylguanine-DNA methyltransferase activity but also a drastic change in their stability and activator functions. Future experiments are being directed to creation of both

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## GENETIC CONTROL OF MUTAGENESIS AND DNA REPAIR

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The *Saccharomyces cerevisiae* *REV1* gene product functions in a cellular process required for mutagenesis caused by ultraviolet (UV) radiation and many chemical mutagens. The *REV1* gene is one of a small group of yeast genes, including *REV3*, *REV7*, *CDC7*, and *NGM2*, whose mutant alleles have a substantial and general effect on mutability. The *REV1* gene product is thought to compose part of a DNA-repair process that is "error-prone" or "mutagenic" in its function. Mutants of *REV1* exhibit little or no mutability either by UV light or by many chemicals, and are only slightly sensitive to the lethal effects of these agents.

Mutagenic repair is distinct from the other major repair pathways in yeast. Excision repair is controlled by genes of the *RAD3* epistasis group, and recombinational strand-break repair is controlled by genes of the *RAD52* epistasis group. The *REV1* gene is part of the *RAD6* epistasis group; the *RAD6* gene product has recently been identified as a ubiquitin transferase that may be required to process histones on chromatin to initiate DNA repair.

As an initial step toward understanding the regulation and function of mutagenic repair, we sought to clone and characterize the *REV1* gene. We plan to use such a clone to overproduce the *REV1* gene product to facilitate purification, characterization, and *in vitro* reconstruction of the mutagenic repair complex. In this regard, other investigators have reported the cloning of the *REV3* gene; a fragment which complements *rev2* has been identified; and *CDC7*, which potentiates mutability when present at high copy number, has been shown to encode a protein homologous to protein kinases.

We have determined the sequence of a 3,341 base-pair segment of DNA that complements the *rev1-1* mutant. Gene disruption was used to confirm that this DNA contained the *REV1* gene. The sequenced segment contains a single long open reading frame (ORF), which can encode a polypeptide of 799 amino acid residues. The *REV1* transcript is 2.6 kb in length. A base substitution, encoding a Gly-to-Arg change, is found in this ORF in *rev1-1*. Frameshift mutations introduced into the ORF yield a *Rev<sup>-</sup>* phenotype. Deletion mutants, lacking segments of the 5' non-coding region of *REV1*, have intermediate mutability relative to *REV1* and *rev1-1*. An in-frame fusion of the 5' end of the *REV1* ORF to the *lacZ* gene produces  $\beta$ -galactosidase activity constitutively. The predicted *REV1* protein is hydrophilic, with a predicted pI of 10.19. No homologies to UMUC, MUCB, *RAD1*, *RAD2*, *RAD3*, *RAD7*, or *RAD10* DNA-repair proteins were noted.

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#### DNA REPAIR AND MUTAGENESIS

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DNA *in vivo* is constantly subject to alterations both spontaneous as well as those induced by exogenous chemical and physical agents. A variety of biochemical processes have evolved in all organisms to repair these damages. However, the repair is often neither perfect nor complete. The persistence of unrepaired lesions, as well as error-prone repair, lead to mutations and possibly tumor induction.

#### DNA Repair

We have been involved in several aspects of DNA repair in human cells and in *E. coli* and in the molecular basis of mutagenesis in human cells. The repair studies involve exclusively removal of alkylation damages in DNA. These include repair of the primary mutagenic lesion, *O*<sup>6</sup>-alkylguanine and the glycolytic removal of *N*-methylpurines, namely, 3-methyl adenine, 3-methylguanine and 7-methylguanine. *O*<sup>6</sup>-Alkylguanine in DNA is repaired by *in situ* dealkylation in a stoichiometric and suicide reaction by *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) which accepts the alkyl group in one of its cysteine residues. MGMT is present in bacteria and in animal tissues and is highly regulated in all systems. Our studies on *E. coli* MGMT have been described elsewhere in this volume in the Protein Engineering report.

The studies on human MGMT repair have included the following aspects. (1) Purification and properties of the protein. (2) Elucidation of various facets of regulation of MGMT both in human cells and mice including tissue-specificity, cell-cycle dependence, and age. (3) Expression of human MGMT in MGMT-negative Chinese hamster ovary (CHO) cells. These transfectant cell lines along with the control CHO cells are being utilized to determine the contribution of *O*<sup>6</sup>-alkylguanine in toxicity, mutagenesis, and chromosome aberrations. (4) The transfectant lines are also being used in the project to clone the human MGMT gene and cDNA.

Finally, the availability of a number of inbred mouse stocks have provided the opportunity to investigate the stock-dependent variation of *N*-methylpurine-DNA glycosylase (MAG) levels in various mouse tissues along with similar studies on MGMT. The results show that the tissue-specific levels of the two enzymes are significantly different. For example, the stomach among all the tissues tested, has the highest level of MAG while MGMT level is the highest in liver, as has been already observed in other animals as well.

### Mutagenesis

Our approach to study the mechanism of mutagenesis and the various factors that affect mutations in human cells is to use a surrogate system, a recombinant shuttle plasmid carrying a target DNA. The mutations in the target that can be retrieved and analyzed should be analogous to the mutations in the host cell that harbors the plasmid. In the experiments carried out so far, we have used an SV40-based vector carrying the *E. coli* *supF*-tRNA gene as the target. While the target has no activity in the human cells, progeny of the mutagenized plasmid molecules following replication in human lymphoblastoid cells are transferred to an appropriate *E. coli* host when the mutation in the target can be phenotypically screened. The mutations are then characterized by the direct sequencing.

In our studies on the mutagenesis by the alkylating agent, *N*-methyl-*N*-nitrosourea (MNU), we have mutagenized only the target sequence and ligated it back to the vector before transfection of the human cells. We compared the mutational yield and type obtained from both MGMT-positive ( $\text{Mex}^+$ ) and MGMT-negative ( $\text{Mex}^-$ ) host cells. The  $\text{Mex}^-$  cells showed about ten-fold higher frequency of spontaneous mutations but only a small increase in induced mutations than the  $\text{Mex}^+$  cells. However, the location and the type of mutations are not significantly different in the two host cells. We have observed a mutational hot spot of the middle G in a GGG sequence that was also observed by others with different mutagens.

We have also been using the same plasmid for studying mutations induced by ionizing radiation. Our immediate objectives are to elucidate not only the types of mutations, excluding large deletions, induced by ionizing radiation but also to establish the effects of a variety of factors on the quality and yield of mutations. These include the following. (1) Effects of the LET of radiation. We plan to compare  $\alpha$ -particles, neutrons and X rays as mutagens. (2) Role of free radical scavengers. (3) Repair competence of the host cells. (4) Difference between split dose and single dose of ionizing radiation exposure.

The mutations induced by  $\alpha$ -particles have not been investigated before at the molecular level. We have extensively studied the mutants induced by X ray which should provide a basis for the subsequent studies with the  $\alpha$ -particles. The mutational hot spots for X ray are significantly different from those induced by MNU. Furthermore, X ray

induces a high proportion of multiple mutations and small deletions including single base deletions.

Our long-term objective is to validate the use of shuttle plasmids to study mutations in host cells by comparing its mutations with those in an appropriate target in the host cells. At the same time, the mutations can be correlated with ionizing radiation-induced chromatid aberrations.

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**STRUCTURE AND ORGANIZATION OF A EUKARYOTIC GENOME  
WITH EMPHASIS ON SATELLITE DNAs, AND  
THE MOLECULAR BIOLOGY OF CRUSTACEAN MOLTING**

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We are defining the organization and function of very highly repeated DNA in the genome of a higher eukaryote, the Bermuda land crab, *Gecarcinus lateralis*. One such DNA has a very complex sequence comprised of two distinctly different classes of domains. Some domains contain unusual primary sequences that provide high-frequency sites for mutations; other domains contain sequences typical of those which harbor genetic information. In exploring the model system, we relate it to the biologically pertinent events of the model animal's changing life cycle.

**Structure, Organization and Function of a Very Highly Repeated DNA**

The DNA of higher organisms including man contains "hotspots" where mutations occur at unusually high frequencies. Such sites are often close to stretches of DNA that are susceptible to changes in the ordinarily regular helical structure of the DNA molecule and are thus susceptible to endonucleases. We are studying such hotspots in a naturally occurring DNA that serves as a model for the DNAs of other higher organisms. This is a so-called satellite DNA, a family of very similar sequences that occur in thousands of copies in the land crab genome. The basic sequence of the satellite repeat unit is of medium length (~2.1 kbp) and complexity compared to known gene sequences. Its multiple repetitions make it favorable material for quantitatively analyzing the subsequences where mutations have arisen in evolutionary history. Our analyses of a number of cloned satellite variants show that major sequence changes occur in regions where the composition of the DNA has certain properties that permit the adoption of an unusual higher order structure. These include: (1) alternating purines and pyrimidines; here, the DNA structure can assume a zig-zag "Z-conformation" and there is a correlated tendency for nearby regions of the molecule to have suffered deletions at a specific site; (2) long tracts of polypyrimidines•polypurines; here, the DNA either undergoes strand slippage or assumes a triple stranded structure and in such regions there are deletions as large as 108 bp in some of the satellite variants.

While the domains of the satellite described above are sites for mutations, other domains have the characteristics of genes and RNA transcripts from them have been identified. Furthermore, many of the transcripts have characteristics of pol II transcription products: they are polyadenylated, strand-specific and tissue- and developmental-stage specific. At a time during the premolt period when the midgut gland (hepatopancreas) atrophies there is a marked decrease in its synthesis of

RNA. Finally, copies of the sequence organized in tandem arrays (satellites) are methylated, commonly seen for untranscribed DNAs, while copies of the sequence dispersed in main-component DNA are not. It may be the latter that are transcribed.

### The Molecular Biology of Crustacean Molting

We use an *in vitro* incubation system for crab tissues developed in this laboratory to study the molecular action of arthropod hormones on protein synthesis. The *in vitro* system is a meaningful model of hormone-induced developmental events which provides information about the mechanism of specific regulation of the molting process in individual organs. 20-Hydroxyecdysone (20HE, arthropod molting hormone) affects protein synthesis of integumentary tissues, ovary and midgut gland very differently. When 20HE *in vivo* induces an animal to enter premolt, integumentary tissues synthesize many proteins associated either with the degradation of the old exoskeleton or with the formation of a new exoskeleton while in the atrophying midgut gland protein synthesis is greatly reduced. These results are duplicated *in vitro*: while 20HE stimulates protein synthesis in integumentary tissues, it inhibits synthesis of most proteins in the midgut gland. Other data suggest that methyl farnesoate (MF, a precursor of the juvenile hormone [JH] of insects) may act as a "crustacean JH" regulating metamorphosis and/or reproduction. Others have shown that MF is secreted by the crustacean mandibular organ (whose function has not been determined previously) and rates of secretion in females are correlated with stage of ovarian development. Trans-trans MF (the natural isomer in crustaceans) as well as JH significantly stimulates total protein synthesis and the synthesis of specific proteins by crab integumentary tissues *in vitro*; the unnatural cis-trans isomer does not. These results provide evidence for a direct (and specific) action of MF and JH on crustacean tissues.

Proteins from specific layers of the exoskeleton from crabs from three different families have been analyzed by SDS-PAGE and 2-D electrophoresis. Two of four proteins extracted from the epicuticle of exuvia of *G. lateralis* are similar in size (45 and 56 kDa) to proteins whose synthesis increases during the stage of proecdysis when epicuticle is formed. A 64 kDa protein extracted from the complete exuvia of one species and the calcified layer of the exuvia of two other species is similar to a protein extracted from the integumentary tissues of *G. lateralis* that cross-reacts with antiserum to insect larval cuticle proteins. These interspecific and inter-class correspondences are being closely investigated.

In addition to studies on the synthesis of proteins, we have extracted from integumentary tissues several alkaline and several acid proteinases that degrade the crustacean exoskeleton during premolt. Confirming the biological function of these proteinases *in vivo* is the absence from the exuviae of newly molted crabs of a series of proteins of the same size as those that are degraded by these proteinases *in vitro*.

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## ENZYMES INVOLVED IN RNA TURNOVER AND SYNTHESIS

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Regulation of the cytoplasmic stability of mRNAs has been found to be a major control mechanism which regulates mRNA levels in a variety of eukaryotic and prokaryotic systems. The degradation rate of an mRNA is important not only in regulating its steady-state level but also in controlling the speed with which changes in the transcription rate are reflected in changes in its cytoplasmic level. An important part of unravelling the mystery of mRNA turnover is to analyze in detail the enzymes that may be involved. Studies of ribonucleases and their possible role in RNA turnover and processing have been continued in this laboratory.

### 5'→3' Exoribonuclease

Studies involving detection, purification, and characterization of unique exoribonucleases of *Saccharomyces cerevisiae* and human placenta have been completed. The enzymes hydrolyze RNA in a 5'→3' mode with the formation of 5'-mononucleotides. mRNA capped with m<sup>7</sup>Gppp is not degraded,

and both enzymes show a stringent specificity for substrates with a 5'-phosphate end group. Their mechanism of action suggests that they may play a role in mRNA turnover in association with an mRNA decapping enzyme such as is described below.

### Decapping Enzyme

Further purification and characterization of a unique enzyme of *Saccharomyces cerevisiae* which was first found in this laboratory and which decaps mRNA to yield m<sup>7</sup>GDP and mRNA with a 5'-phosphate end group have been carried out. Others have shown that capped mRNAs are more stable, that the cap structure may be required for splicing, and that capped mRNAs are much more efficient in stimulating translation. It follows then that an enzyme that removes the cap structure could cause severe inactivation of mRNA, as well as render it more susceptible to degradation. The enzyme may be involved in mRNA turnover or, possibly, used by the cell to modulate the efficiency of protein synthesis. Purification studies show that in the early steps of isolation the enzyme is closely associated with an activity which is similar to the 5'→3' exoribonuclease described above. It can be separated from the exoribonuclease by heparin-agarose chromatography, a step which yields an additional fivefold purification. The total purification of the decapping enzyme is 25,000-fold. The specificity of the enzyme has been studied using both yeast mRNA and synthetic RNAs labeled in the cap structure. A synthetic capped RNA with a chain length of 540, made with the bacteriophage SP6 polymerase and vaccinia virus capping enzyme, is 75% decapped by the purified enzyme, but the RNA is essentially unaltered in size. Small oligoribonucleotides containing the cap structure are not substrates. Concentrations of RNase A which degrade the RNA to an average chain length of 50 or less reduce the rate of decapping by more than 70%. Hydrolysis rates of synthetic capped RNAs of different sizes (50 to 540 nucleotides in length) show that the larger RNA can be as much as 10 times better as a substrate. Capped RNA lacking a 7-methyl group on the Gppp was also synthesized with the vaccinia virus capping enzyme, and it was found to be hydrolyzed at a rate similar to that of the capped RNA containing such a group. The pyrophosphate bonds of the triphosphate end group of uncapped RNA synthesized with RNA polymerase of *Escherichia coli* are not detectably hydrolyzed. Hydrolysis of capped poly(A) was not measurable. The unique specificity of the decapping enzyme for long capped RNA chains makes it a highly specific pyrophosphatase.

### RNA Polymerase II

Studies have been initiated to investigate the role that phosphorylation of RNA polymerase II plays in the control of the rate of transcription. Others have shown that RNA polymerase II phosphorylated on the repeated heptapeptide sequence found at the carboxyl terminus of the largest subunit is the most active form of the enzyme. Our investigation is centered on the extent of phosphorylation of the carboxyl terminus under different growth conditions and on determinations of the rate of turnover of the phosphate groups. Also, protein kinases which

phosphorylate the carboxyl terminal sequence are being investigated. Toward these ends, a synthetic peptide containing 28 amino acids (4 repeats) of the sequence is being used. An antibody for the peptide has been obtained by immunization of rabbits with it; the antibody is being further characterized. The peptide has also been used as a substrate in a search for protein kinases which phosphorylate it. Fractionation of HeLa whole cell lysates shows the presence of a kinase which actively phosphorylates the peptide and which can be separated from casein kinases I and II. The kinase activity is inhibited strongly by 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole, which specifically inhibits mRNA synthesis in mammalian cells. The only other protein kinase which has been found to be inhibited by the same nucleotide analog is casein kinase II. Studies are in progress to determine the specificity of the HeLa protein kinase.

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#### STRUCTURAL BIOLOGY OF NUCLEOSOMES AND GENES

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This laboratory is investigating the structure of macromolecular components in genetic material. The goal of this research program is to understand the structural organization, function, and dynamic behavior of the nucleosome, DNA, histones, chromatin, and other macromolecules in relation to control of gene expression.

#### Nucleosome Core Particle Crystal Structure

The nucleosome core particle structure has been determined to a resolution of 8 Å by X-ray crystallographic methods. Features present in this structure include the histone organization, the double helical DNA structure, numerous protein-DNA interactions, and distortions in the path

of the DNA around the protein core. The DNA in the nucleosome core particle bends much more substantially than any other DNA structure solved by crystallographic methods. The size and shape of the DNA grooves provide evidence of the distortions in the B-DNA structure which facilitate DNA bending. Our research indicates that the bending of DNA is accomplished by considerable compression of the major grooves on the inner surface next to the protein core and expansion on the outside of the superhelix. We have also detected a structural motif in the DNA with a period of two B-DNA helical repeats, or about 20 base pairs. This feature results from the placement of AA and TT dinucleotides at specific positions within the 20 base pair repeat, and these dinucleotides modulate the DNA structure for optimum major and minor groove bending around the histone core. Nucleosomes reconstituted from homogeneous histone octamers and cloned specific-sequence DNA are necessary in order to determine a high resolution ( $\sim 3$  Å) crystal structure. Several DNAs that form precisely positioned nucleosomes are being developed, including a tRNA gene complete with its polymerase promoter region. The high resolution structure will be phased using multiple energy anomalous dispersion data collected at the Brookhaven National Synchrotron Light Source. It will be possible to trace the peptide chains of the histone molecules and the phosphodiester backbone of the DNA, and locate the DNA bases in the resulting electron density map. This long term research is providing information to understand control of gene expression at the nucleosomal level.

#### **Structural Studies of DNA**

Sequence dependent DNA curvature or bending appears to mediate recognition of sequences by regulatory proteins, and also influences the location of precisely positioned nucleosomes. The structural origins of DNA curvature and bendability are being investigated by computer modeling using energy minimization techniques. The positional preferences of dinucleotide types in precisely positioned nucleosomes have been analyzed by statistical methods and algorithms developed for the prediction of nucleosome positions along DNA. Analyses of the DNA sequence patterns in precisely positioned nucleosomes have revealed the existence of several DNA sequence periodicities including 6-7, 10, and 21 base pairs. The results demonstrate that each dinucleotide type is unique in terms of its positional preference in precisely positioned nucleosomes, and that the sequences adjacent to major groove-in sites are of paramount importance for bending into the major groove. The positional preferences of AA and TT dinucleotides used with the roll/tilt wedge model of DNA bending predict the 20 base pair structural repeat observed in the crystal structure of our nucleosome core particle.

#### **National Center for Small-Angle Scattering Research**

The National Center for Small-Angle Scattering Research, located at Oak Ridge National Laboratory, provides sophisticated instrumentation for neutron and X-ray scattering research in biology. Scattering research on nucleosomes, histones, chromatin, and other macromolecules is under way at

this facility. The functional relationships between structural elements in shear-aligned 30 nm chromatin fibers are being investigated by neutron scattering. This research will provide details about the arrangement of nucleosomes in chromatin, as well as insights into the structural determinants of actively transcribing genes compared to inactive genes. The repair enzyme *O*<sup>6</sup>-methylguanine-DNA methyltransferase is also being studied by X-ray and neutron small-angle scattering in support of efforts to obtain crystals suitable for X-ray crystallography. The radius of gyration of this enzyme is 20 Å and the scattering curve is best approximated by a globular model.

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## CRYSTAL STRUCTURE STUDIES ON SMALL ORGANOPLATINUM COMPOUNDS

C. H. Wei

Platinum complexes have not only revealed several structural principles of inorganic coordination chemistry, but some of them, such as *cis*-diaminodichloroplatinum(II) have found application as antitumor drugs. Crystallographic studies on some platinum-nucleosides and platinum-nucleotides have also been reported. Although the formation of tetra(pyridine)platinum(II) chloride has been mentioned in the literature, the degree of hydration of this compound and its crystal structure were not given. Our isolation of this compound not only permitted our determination of its structure and the number of water molecules in a formula unit but has also enabled us to demonstrate the three-dimensional network of its hydrogen bonding scheme by the refinement based on combined X-ray and neutron scattering data.

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1. Wei, C. H. Structure of ammonium methanesulfonate. *Acta Crystallogr.* **C42**: 1839-1842, 1986.
  2. Wei, C. H. Structures of two isomers of phenyl-3*H*-1,2-dithiole-3-thione. *Acta Crystallogr.* **C42**: 1836-1839, 1986.
  3. Wei, C. H. Structure of 4-methyl-5-[2-(2-pyrazinyl)vinyl]-3*H*-1,2-dithiole-3-thione. *Acta Crystallogr.* **C43**: 113-115, 1987.
  4. Wei, C. H. Structure of polymeric pyridinium pentachlorodidcadmate(II). *Acta Crystallogr.* **C43**: 2295-2298, 1987.
  5. Wei, C. H., B. H. Hingerty, and W. R. Busing. Structure of tetra(pyridine)platinum(II) chloride: unconstrained anisotropic least-squares refinement of hydrogen-nonhydrogen atoms from combined X-ray-neutron diffraction data. *Acta Crystallogr.* **C44**: in press.
  6. Wei, C. H., B. E. Suttle, and B. C. Pal. Structure of the hydrate form of 3-methyl-2,6,7,8-tetrahydro-2-oxo-3*H*-pyrimido[5,4-*b*]1,4-thiazine-7-carboxylic acid. *Acta Crystallogr.*, in press.

**REGULATION OF MAMMALIAN-CELL TRANSPORT SYSTEMS**

J. S. Cook	P. J. Galloway <sup>4</sup>
T. M. Babbitt <sup>6</sup>	M. R. Hauser <sup>1</sup>
M. H. Bast <sup>4</sup>	C. J. Shaffer <sup>6</sup>
W. D. Dawson <sup>3</sup>	B. G. Stanford

The transport of metabolites across either cell surfaces or epithelial sheets of cells is studied by this group in model mammalian cell systems. Cloned cells in culture are used because they insure physiological and genetic homogeneity as well as experimental control over the chemical environment (hormones, growth factors), thus permitting the unambiguous identification of cell types and the environmental factors to which they respond. Recent work has focused on 2 kidney cell lines that differentiate *in vitro*: LLC-PK<sub>1</sub>, proximal tubule cells derived from pig kidney, and MDCK, distal tubule cells derived from dog kidney. The goal of this group is to develop an understanding of the regulation of specific transport systems in relation to cell growth and differentiation. Three projects are outlined for this report period:

**Protein Kinase C and the Regulation of Amino Acid Transport**

Depletion of endogenous amino acids in confluent LLC-PK<sub>1</sub> cells uncovers a subsequent biphasic response of Na<sup>+</sup>-dependent A-system amino acid transport to the tumor promoter and co-mitogen 12-O-tetradecanoylphorbol 13-acetate (TPA); the early response corresponds to a TPA-induced translocation of protein kinase C from cytosol to the membrane and an increase in membrane protein phosphorylation. Both transport and phosphorylation peak within 5-10 min after TPA exposure and return to near control levels for the next 20-30 min. The increases in transport and phosphorylation can both be inhibited by sphingosine, an inhibitor of protein kinase C, and are prolonged in the presence of NaN<sub>3</sub>, a phosphatase inhibitor. Several membrane-associated proteins show a rise and fall of phosphorylation in response to TPA and sensitivities to sphingosine and NaN<sub>3</sub> corresponding to the observed transport changes. Similar rapid responses are elicited by diglycerides that also activate protein kinase C and stimulate the A-system. In confluent cultures sphingosine inhibits only the TPA-sensitive portion of A-system transport but not the basal rate. Conversely, in rapidly growing cultures in which the kinase is constitutively membrane-associated and which have a >15-fold higher rate of transport, TPA is without effect and sphingosine inhibits the A-system by 50%. The results suggest that membrane-associated protein kinase C maintains the transporter or a transport regulator in a phosphorylated, active state in growing cells, but dissociates from the membrane at confluence, after which time the regulators are dephosphorylated.

The second phase of the TPA response in confluent cells is also initiated within 5 min by a protein kinase C-mediated event but does not

becomes apparent for 45-60 min. Unlike the first phase, the late response is sensitive to RNA and protein synthesis inhibitors. Several TPA-induced proteins correspond to those phosphorylated in the first phase.

#### **Inhibition of Na<sup>+</sup>-Dependent Hexose Transport by Amiloride Analogs**

An outstanding property of LLC-PK<sub>1</sub> cells is their expression in culture of Na<sup>+</sup>-dependent uptake of hexoses, a proximal tubule function. This system is the means by which, *in vivo*, glucose filtered at the renal glomerulus is recovered by the body. The up-hill transport of glucose is energized by its being tightly coupled to the Na<sup>+</sup> gradient between the filtrate and the proximal tubule cell interior. Na<sup>+</sup> transport in other segments of the kidney is blocked by the diuretic amiloride, a compound in clinical use to prevent Na<sup>+</sup> retention, and E. J. Cragoe (Merck, Sharp and Dohme) has synthesized a series of potent analogs to the parent compound. It has now been demonstrated that the analogs can interact directly with the Na<sup>+</sup>-binding site on the hexose transporter in LLC-PK<sub>1</sub> cells and block sugar uptake. The pharmaceuticals thus have the potential *in vivo* not only of elevating Na<sup>+</sup> excretion by interfering with Na<sup>+</sup> reabsorption at a hitherto unsuspected site, but also of inducing urinary loss of glucose.

#### **Transport of Polyamines**

Polyamines are a group of compounds including putrescine, spermidine, and spermine, containing two to four amino groups. They are required for cell growth and differentiation, possibly through an interaction with exposed phosphate groups in DNA. In both kidney lines studied, polyamines are taken up by a transporter that is separate from the well-known organic base transporter. Uptake is dependent on Na<sup>+</sup>. Polyamines are transported across sheets of cells in a direction suggesting that *in vivo* they are recovered from the glomerular filtrate and restored to circulating blood plasma. A provocative finding is that the aminoglycoside antibiotics gentamicin and kanamycin, which are toxic to the kidney, compete with and can block polyamine uptake by kidney cells.

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1. Cook, J. S. "Spark" vs. "Soup": A Scoop for "Soup." *News Physiol. Sci.* 1: 206-208, 1986.
  2. Cook, J. S. Sectionalization of the society. In: *History of The American Physiological Society: The First Century, 1887-1987*, ed. by J. R. Brobeck, O. E. Reynolds, and T. A. Appel. American Physiological Society, Publ., Bethesda, Maryland, 1987, pp. 435-461.
  3. Cook, J. S., C. Shaffer, and E. J. Cragoe, Jr. Inhibition by amiloride analogs of Na<sup>+</sup>-dependent hexose uptake in LLC-PK<sub>1</sub>/CL4 cells. *Am. J. Physiol.* 253 (*Cell Physiol.* 22): C199-C204, 1987.
  4. Dawson, W. D., and J. S. Cook. Parallel changes in amino acid transport and protein kinase C localization in LLC-PK<sub>1</sub> cells treated with TPA or diradylglycerols. *J. Cell. Physiol.* 132: 104-110, 1987.

5. Dawson, W. D., and J. S. Cook. Biphasic response of  $\text{Na}^+$ -dependent amino acid transport to tumor promoting phorbol esters in cultured renal epithelial cells, LLC-PK<sub>1</sub>. In: *Membrane Biophysics III: Biological Transport*, ed. by M. Dinno and W. McD. Armstrong. Alan R. Liss, New York, 1988, pp. 121-131.

### THEORETICAL AND APPLIED CRYOBIOLOGY

P. Mazur  
U. Schneider<sup>6</sup>

K. W. Cole  
J. W. Hall

#### Basic Mechanisms of Slow Freezing Injury

The cause of slow freezing injury and the basis of the protection by solutes like glycerol are the subject of debate. During slow freezing, cells are sequestered in unfrozen channels between ice crystals that grow by pulling pure water out of the channels. As a consequence, the solute concentration in the channels rises and the channels progressively shrink in size. The rise in solute concentration, in turn, causes the cells to progressively shrink osmotically. Until recently cryobiologists have ascribed slow freezing injury either to the rise in solute (electrolyte) concentrations or to the consequent cell shrinkage, rather than to the size of the channels. But over the past two years we have demonstrated that an equally or even more important cause of injury is the crowding of human red blood cells into ice-lined liquid channels of progressively smaller dimensions. We believe that the damage may result from the deformation of cells or from shear-stress forces in these narrowing channels. It may also result from intimate cell-cell contacts produced by the crowding. One speculation is that cell membranes within a deformed cell or between cells undergo actual fusion. Three lines of evidence support these views:

1. Although ordinarily reciprocally coupled, it is possible to separate the composition of the unfrozen channels from their size, or more precisely from the size of the unfrozen fraction, by suspending cells in NaCl/cryoprotectant solutions in which the mole ratio of the two is held constant, but the molality of the NaCl is allowed to vary. When human red cells are frozen in such solutions to temperatures that produce given NaCl concentrations ( $m_s$ ), but varying unfrozen fractions ( $U$ ), survival at low  $U$  is found to be strongly dependent on  $U$  but independent of  $m_s$ . Only at higher values of  $U$ , does survival become inversely dependent on both  $m_s$  and  $U$ .

2. Orthodox theory holds that slow freezing damage results from the sequential exposure of cells to high solute concentrations during freezing followed by solute dilution during thawing. We, however, have recently

determined that when the ice formed during freezing is dissolved at subzero temperatures without the occurrence of solute dilution, the damage to human red cells at low unfrozen fractions is still evident.

As a control, cells were subjected to comparable temperature, times, and solute concentrations, but without freezing. In this case, when the unfrozen fraction is 1, survival was 90%. When freezing was allowed to occur, survival was 40% or less at low values of unfrozen fractions.

3. As the unfrozen fraction decreases during freezing, the cells become crowded together and likely contact each other while they also become progressively shrunken osmotically from the increasing solute concentrations in the unfrozen channels. To mimic these effects, we have subjected human red cells to close contact in unfrozen medium by centrifugation. Damage is extensive when the cells are shrunken osmotically and centrifuged at accelerations  $> 5000 \times g$ , and at subzero temperatures. In the absence of any one of these, little or no damage occurs.

The view that a major component of freezing injury is the crowding of shrunken distorted cells into ever narrowing unfrozen channels may be especially important in the freezing of multi-cellular tissues and organs in which cells are already "crowded together." Although largely unsuccessful to date, the successful freezing of multi-cellular tissues and organs would have important clinical applications, especially in transplantation.

#### **Mouse Embryo Banking**

There are several reasons to preserve mouse genetic stocks in the form of frozen embryos: (1) As insurance in case the breeding stock is lost by reproductive failure, disease, or fire (which occurred at Jackson Labs in 1947). (2) To study, control, or reduce genetic drift in inbred lines. Since an embryo at  $-196^{\circ}\text{C}$  remains unchanged for centuries, it can be used as an invariant standard against which to assess genetic drift. Frozen embryos can also be used to regenerate inbred lines periodically. (3) To permit experiments that otherwise could not be undertaken, such as a comparison on the same day between a pair of monozygotic twins that differ in age. (4) To provide a method of maintaining genetic stocks that is cheaper than conventional breeding in terms of manpower, space, and facilities.

We are engaged in three embryo banking projects funded by other agencies through interagency agreements. One through the National Institute on Aging involves the banking of embryos from special inbred lines maintained by NIA. One through the NIH and Oregon Health Sciences University involves the banking of mouse embryos at various stages in the genetic selection of lines showing various sorts of abnormalities in alcohol metabolism. The third through the National Institute on Child Health and Human Development involves the freezing of 2000 embryos in the 8-cell precompaction stage. These projects are an instance of the

transfer of a technology first developed in this laboratory in 1972. Since this work parallels the mouse embryo banking carried out in the Division by L. B. Russell, it keeps us in a position to assist her in trouble-shooting problems and to advise her on developments in the field of banking.

### **Cryobiology of *Drosophila* Embryos**

The early embryos of mammals exist in the closely controlled environment of the uterus. The early embryos of oviparous animals like insects are subject to wide ranges in temperature, humidity, and other aspects of the environment. Yet paradoxically, mammalian embryos can now be frozen with survivals approaching 100% while those of insects and most other non-mammalian groups have so far totally resisted all attempts. Aside from the fundamental question of what is responsible for the difference, a practical point is that the ability to bank *Drosophila* embryos at low temperatures in a fashion analogous to mouse embryos, would be of substantial benefit to *Drosophila* geneticists who currently are forced to maintain some 10,000 mutant lines by standard breeding and monthly transfers. With support from a grant from the National Science Foundation and in collaboration with K. B. Jacobson of this Division, and A. P. Mahowald of Case Western Reserve University we are investigating the problem.

One fundamental way in which the *Drosophila* egg differs from the mouse egg is that it is isolated from the outside environment by a waxy layer in the vitelline membrane that allows gas and vapor exchange but not liquid water or solutes. Standard freezing procedures require the presence of a protective solute like glycerol that penetrates the cell, and also require that the cell be able to lose water osmotically during freezing so as to preclude intracellular freezing. The approaches of others to date have been to try to convert the *Drosophila* embryo into the standard situation by removing the wax in the vitelline layer. This permeabilizing requires placing the eggs in octane, a drastic treatment that so far has not produced survivals after freezing to  $-20^{\circ}\text{C}$  or below. Before pursuing this approach, we opted to examine the water permeability and resistance to dehydration and low temperature of the intact, unpermeabilized egg. The result of these studies indicated that orthodox approaches to freezing cells will probably not work for *Drosophila* eggs. The orthodox procedure is to freeze cells slowly enough to avoid intracellular freezing. But our work with intact eggs has shown that even in the absence of any freezing, the eggs become increasingly sensitive to progressively lower subzero temperatures, and by  $-20^{\circ}\text{C}$  they die in a matter of minutes. Consequently, slow cooling to prevent intracellular freezing will result in their death from low temperature per se.

Another approach is to prevent both the low temperature injury and intracellular freezing by cooling the eggs extremely rapidly to temperatures below which any adverse reactions can occur; i.e., to below  $-120^{\circ}\text{C}$ . Under special conditions, rapid cooling converts water in cells

into a glass rather than into ice. These special conditions include permeating the cell with a solute, like DMSO, that promotes vitrification.

Penetration of glass-inducing agents into *Drosophila* eggs requires their permeabilization; e.g., by removal of the waxy layer with octane. We have developed a reliable method for so doing that entails passing the required fluids through eggs sandwiched between Nuclepore filters. The next problem is to achieve high and consistent viability of the permeabilized eggs; others have reported obtaining high survival (80%).

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1. Mazur, P. Stopping biological time - the freezing of living cells. *Ann. NY Acad. Sci.*, in press
  2. Mazur, P., and K. W. Cole. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. *Cryobiology*, in press.
  3. Schneider, U., and P. Mazur. Relative influence of unfrozen fraction and salt concentration on the survival of slowly frozen eight-cell mouse embryos. *Cryobiology* 24: 17-41, 1987.

# **Educational Activities**

## **Postdoctoral Training Program**

Postdoctoral training is an important feature of Division activities, providing benefits both to the trainees and to the Division. Support for these training activities is derived from a variety of sources and is administered by The University of Tennessee and other universities. Two sources of funds are a subcontract from Martin Marietta Energy Systems, Inc., and a postdoctoral training grant in Carcinogenesis from the National Cancer Institute. Some appointments are also made through Oak Ridge Associated Universities (ORAU). During the past two years, there were 13 trainees enrolled in these postdoctoral programs. After a two- or three year period of research in the Biology Division, trainees obtain positions in universities, industries, or other government laboratories.

## **Doctoral Training Program**

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences is located within the Biology Division of Oak Ridge National Laboratory. The program is primarily designed to provide high quality, multidisciplinary graduate education and research training leading to the Ph.D. degree, although there are a few Master's degree candidates. Ph.D. students are supported by The University of Tennessee in the form of research assistantships or by federal training grants awarded to the School by the National Institutes of Health. The School currently has 28 students working the Ph.D. degree. As of August, 1988, 137 students have been awarded the Ph.D. or M.S. degree.

The School celebrated its 20th anniversary in October, 1986; a mini-symposium comprising 16 papers presented by former graduates was held at the Biology Division. R. A. Popp is the Acting Director. The School has a faculty of two full-time professors, Daniel Billen and Donald Olins, and three research professors, Ada Olins, Robert Foote, and Ed Uberbacher. The major portion of the School's teaching and research training is provided by 29 adjunct faculty members; 24 of the adjunct faculty are members of the Biology Division.

The students form a very active group of investigators in training, and their names appear on 24 publications, 16 as first authors, during the past two years. This represents a significant contribution to the productivity and excellence of ORNL's Biology Division. In return, the students receive superb guidance and training by staff members of the Biology Division.

## Undergraduate Training Programs

The Biology Division participates in three undergraduate training programs: (i) Great Lakes Colleges Association/Associated Colleges of the Midwest (GLCA/ACM Science Semester), (ii) Southern Colleges University Union Science Semester (SCUU), and (iii) Oak Ridge Associated Universities Summer Student Trainee (ORAU). Under the auspices of these organizations and in cooperation with Oak Ridge National Laboratory, outstanding college juniors are offered opportunities for independent research in the life sciences. In the past 24 months, there were 25 students, possessing the educational qualifications and the potential for a successful scientific career, who spent 16 weeks (GLCA/ACM and SCUU) or 10 weeks (ORAU) performing research under the guidance of Biology Division staff members.

Although the principal purpose of the programs is to provide a training experience for the students, it often allows Division staff members an opportunity to broaden their areas of research. Upon completion of their research activities in the laboratory, students prepare a formal scientific paper and present a talk on their work. The programs, in which over 500 students have participated during the past 20 years, have received the enthusiastic endorsement of the students, their colleges, and the members of the Biology Division.

## Training Grants

Title	Awarded by	Principal Investigator
Predoctoral Training Program in Genetics	National Institute of General Medical Sciences	R. A. Popp
Predoctoral Training in Carcinogenesis Research	National Cancer Institute	F. T. Kenney
Postdoctoral Training in Carcinogenesis Research	National Cancer Institute	F. T. Kenney
Graduate Training in Radiation Biology	National Cancer Institute	M. Terzaghi

## Appendices

### Advisory Committee - FY 1988

Dr. Robert L. Dixon  
Director of Toxicology  
Sterling-Winthrop Research Institute  
Columbia Turnpike  
Rensselaer, New York 12144

Dr. Lorraine Flaherty  
Chief, Laboratory of Immunology  
Wadsworth Center for Laboratories & Research  
New York State Department of Health  
Empire State Plaza  
Albany, New York 12201

Dr. William J. Schull, Director  
Center for Demographic & Population Genetics  
The University of Texas Health Science Center  
Post Office Box 20334  
Houston, Texas 77225

Dr. Shirley M. Tilghman  
Howard A. Prior Professor of the Life Sciences  
Department of Molecular Genetics  
Princeton University  
Princeton, New Jersey 08544

Dr. Joseph J. Villafranca  
Professor  
Department of Chemistry  
152 Davey Laboratory  
Pennsylvania State University  
University Park, Pennsylvania 16802

Dr. Arthur Weissbach  
Associate Director  
Department of Cell Biology  
Roche Institute of Molecular Biology  
Kingsland Street  
Nutley, New Jersey 07110

## Extramural Activities

### Society Committees

- J. S. Cook - Publications Committee, American Physiological Society, 1986-1989
- R. J. M. Fry - History Committee, Radiation Research Society, 1982-
- P. Mazur - Publications Committee (Chairman), Society for Cryobiology, 1974-  
Board of Governors, Society for Cryobiology, 1981-
- A. L. Olins - Constitution and By-laws Committee, American Society for Cell Biology, 1982-1987  
Councilor, American Society for Cell Biology, 1984-1986  
E. B. Wilson Award Nomination Solicitation Committee, American Society for Cell Biology, 1984-1986 (Chairperson, 1986)  
Minorities Committee, American Society for Cell Biology, 1985-  
Program Committee, Electron Microscopy Society of America, 1986
- R. J. Preston - President-elect, Environmental Mutagen Society, 1988-1989  
Future Directions Committee, Environmental Mutagen Society, 1988-1989  
John Sealy Memorial Endowment Fund Grants Committee, University of Texas Medical Branch, 1987-
- L. B. Russell - Committee on Future Meetings, Environmental Mutagen Society (Chairman, 1986-1987)  
Workshop organizer, Environmental Mutagen Society, 1986-1987
- D. M. Skinner - Board of Governors (North America), The Crustacean Society, 1987-  
Selection Committee, Miller Research Fellows, Miller Institute for Basic Research in Science, University of California, Berkeley, 1987-  
Chair, Nominating Committee, Comparative Physiology and Biochemistry Division, American Society of Zoologists, 1988

- R. L. Ullrich - Finance Committee, Radiation Research Society, 1984-1987  
 Awards Committee, Radiation Research Society, 1987  
 North American Data Effects Group, Chairman, 1980-

#### Advisory Committees

- J. S. Cook - Member of the Corporation, Mount Desert Island Biological Laboratory, 1962-  
 Special Study Sections, National Institutes of Health, 1987, 1988  
 Panel on ONR Research in Biology and Medicine, National Academy of Sciences/National Research Council, 1987
- R. J. M. Fry - Scientific Committee 40, National Council on Radiation Protection and Measurements, 1977-  
 Council Member, National Council on Radiation Protection and Measurements, 1980-  
 Scientific Committee 75 (Chairman), National Council on Radiation Protection and Measurements, 1983-  
 Advisory Committee, Radiological Research Accelerator Facility, Columbia University, 1983-  
 Committee 1, International Commission on Radiological Protection, 1985-1989  
 Advisory Committee, Institute of Environmental Medicine, New York University Medical Center, 1985-  
 Vice-Chairman, Subcommittee F.2 on Radiation Biology, Committee on Space Research, 1986-1988  
 Committee on Space Biology and Medicine, Space Science Board, National Research Council, National Academy of Sciences, 1986  
 Subcommittee on Biological Effects of Diagnostic and Other Low-Level Radiations, American College of Radiology, 1987  
 Member, Aerospace Medicine Advisory Committee, National Aeronautical Space Administration, 1988  
 Chairman, Environmental Sciences Working Group, National Aeronautical Space Administration, 1988  
 Member, Bevalac Biomedical Program Advisory Committee, Lawrence Berkeley Laboratory, 1988-1990

- W. M. Generoso - Subcommittee on the Use of Biological Markers in Reproductive and Developmental Toxicology, National Academy of Sciences, 1987
- R. A. Griesemer - Science Advisory Board, U.S. Environmental Protection Agency, 1984-  
Executive Committee, 1984-  
Chairman, Environ. Health Committee, 1984-  
Health and Environmental Research Advisory Committee (HERAC), Department of Energy, 1985-;  
Chairman, Subcommittee on Radiation Biology, 1985-  
Board of Scientific Counselors, National Toxicology Program, 1987  
Member, U.S.-Japan Joint Environmental Panel, 1986-  
Member, Working Group on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer, Lyon, 1987  
Joint Graduate Coordinating Committee, Comparative and Experimental Medicine Graduate Program, The University of Tennessee, 1984-
- S. J. Kennel - Study Section on Health Effects Research, U.S. Environmental Protection Agency, 1982-  
Ad hoc member, National Cancer Institute Site Visit Committee, 1986
- F. T. Kenney - Adviser, National Cancer Institute Outstanding Investigator Grants Program, 1984-
- A. C. Marchok - Member, Site Visit Committee for National Cancer Institute Grants on Chemopreventive Retinoids, 1987  
Special Reviewer, Pathology B, Study Section, National Institutes of Health, 1988  
Member, Ad Hoc Technical Review Committee for Request for Proposals on Chemoprevention, National Institutes of Health, 1988
- Peter Mazur - Space Science Board Ad Hoc Panel on Mars Sample Return, National Academy of Sciences, 1987-1988
- S. Mitra - Consultant, National Cancer Institute, 1987-
- A. L. Olins - Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-  
Gordon Conference on Chromatin, 1986 (Co-chairman)

- D. E. Olins - Member of the Corporation, Woods Hole Marine Biological Laboratory, 1983-  
Advisory Committee, Helicon Foundation, La Jolla, California, 1983-  
Gordon Conference on Chromatin, 1986 (Co-chairman)
- R. A. Popp - Mouse Hemoglobin Nomenclature, 1984-  
Special Reviewer, Sickle Cell Program Projects, National Institutes of Health, 1987  
Special Reviewer, Mammalian Genetics Study Section, National Institutes of Health, 1988
- R. J. Preston - Cytogenetic Adviser to Ethylene Oxide Council and to Health Industry Manufacturers Association, 1981-  
Genetics Working Group, American National Standards Institute, 1983-  
Talent Pool for Committee on Radiation Research and Policy Coordination, 1985-  
Committee for National Cancer Institute/Oak Ridge Associated Universities on Radiation Biological Dosimetry in Reference to Atomic Veterans, 1985-  
Gene-Tox Panel on In-Vivo/In Vitro Cytogenetics (Chairman), Environmental Protection Agency, 1986-  
Toxicology Study Section, National Institutes of Health, 1988-1992
- L. B. Russell - International Committee on Standardized Genetic Nomenclature for Mice, 1977-  
Coordinating Committee of Gene-Tox, U.S. Environmental Protection Agency, 1980-  
Board on Toxicology and Environmental Health Hazards, National Academy of Sciences, 1981-1986  
Board on Environmental Science and Toxicology, National Academy of Sciences, 1986-1990  
Environmental Health Institute, Fellow, 1987-
- W. L. Russell - Member, Veterans Administration Scientific Review Committee on "Mortality Follow-up Study of Crossroads Nuclear Test Participants"
- G. A. Sega - Subcommittee on Safe Drinking Water/DNA Adducts, National Research Council, 1987-

- P. B. Selby - Task Group XI of National Council on Radiation Protection Committee 57, Genetic Risk from Internal Emitters, 1984-  
Scientific Adviser and Member of the U. S. Delegation to The United Nations Scientific Committee on the Effects of Atomic Radiation, 1986-
- D. M. Skinner - Member of the Corporation, Marine Biological Laboratory, Woods Hole, 1971-  
Panel Member, Regulatory Biology Program, National Science Foundation, 1986-
- A. L. Stevens - Biomedical Sciences Study Section, National Institutes of Health, 1985-
- J. B. Storer - Scientific Committee 75, National Council on Radiation Protection and Measurements, 1983-
- R. L. Ullrich - Scientific Committee 40 on the Biological Aspects of Radiation Protection Criteria, National Council on Radiation Protection and Measurements, 1977-  
Radiation Study Section, National Institutes of Health, 1987-1991  
Invited Participant, NAS/NRC Committee on Biological Health Effects of Ionizing Radiation, 1987-
- W. K. Yang - Experimental Virology Study Section, National Institutes of Health, 1987-1988  
Cancer Biology and Immunology Contract Review Committee, National Cancer Institute, 1987-1991  
Outstanding Investigator Award Review Committee, National Cancer Institute, 1987-1988

## Editorial Boards

- J. S. Cook - *American Journal of Physiology*  
Associate Editor, 1981-1987; Acting Editor,  
1984-1985; Editorial Board 1987-1990  
*Cell and General Physiology*, American  
Physiological Society Handbook Series,  
1982-  
*Current Topics in Membranes and Transport*  
Advisory Board, 1983-  
*News in Physiological Sciences*  
Editorial Board, 1985-1986  
Associate Editor, 1986-1989
- R. J. M. Fry - *Radiation Research*, Editor-in-Chief, 1988-
- W. M. Generoso - *Teratogenesis, Carcinogenesis, and*  
*Mutagenesis*, 1979-  
*Mutation Research*, 1985-1988  
*Molecular Toxicology*, 1986-
- F. C. Hartman - *BioScience*, 1980-1986  
*Journal of Protein Chemistry*, 1982-1992  
*Journal of Biological Chemistry*, 1983-1988
- P. Mazur - *Cryobiology*, 1967-
- A. L. Olins - *European Journal of Cell Biology*, 1982-1987
- D. E. Olins - *Molecular and Cellular Biochemistry*,  
1983-1986
- R. J. Preston - *Mutation Research*, 1977-  
*Environmental and Experimental Botany*, 1979-  
*Mutation Research Letters*, Managing Editor,  
1980-  
*Teratogenesis, Carcinogenesis, and*  
*Mutagenesis*, 1980-
- J. D. Regan - *Cell Biology and Toxicology*, 1983-
- L. B. Russell - *Mutation Research*, 1976-  
*Mouse News Letter*, 1987-
- G. A. Sega - *Environmental Mutagenesis*, 1985-
- D. M. Skinner - *Growth*, 1979-  
*Gene*, 1986-
- R. L. Ullrich - *Radiation Research*, 1983-1989

**Awards, Honors**

- R. A. Griesemer - Ohio State University, Distinguished Alumnus Award, 1987
- F. C. Hartman, - Martin Marietta Energy Systems, Inc.,  
C. D. Stringer, Publication Award, 1987  
S. Milanez, and  
E. H. Lee
- F. W. Larimer, - Martin Marietta Energy Systems, Inc.,  
E. H. Lee, Publication Award, 1988  
R. J. Mural  
T. S. Soper, and  
F. C. Hartman
- P. Mazur - Two publications designated Citation Classics  
by the Institute of Scientific Information
- A. L. Olins - Eleanor Roosevelt International Cancer  
Fellowship, 1987  
Theodor Boveri Award, University of Würzburg, 1987  
Chancellor's Award for Research and Creative  
Achievement, The University of Tennessee, 1988
- D. E. Olins - American Cancer Society, Scholar in Cancer  
Research Award, 1987  
Theodor Boveri Award, University of Würzburg, 1987
- J. D. Regan - Two publications designated Citation Classics by  
the Institute of Scientific Information
- L. B. Russell - U.S. Department of Energy, Distinguished Associate  
Award, 1987  
Martin Marietta Energy Systems, Inc., Technical  
Achievement Award, 1988
- D. M. Skinner - Women in Cell Biology, American Society of Cell  
Biology, Career Recognition Award, 1987
- R. L. Ullrich - Radiation Research Society, Research Award, 1987

**Abstracts for Technical Meetings Held  
October 1, 1986 - September 30, 1988**

- Adler, Howard I., and Weldon Crow. Genetic instability in *Clostridium acetobutylicum*. American Society for Microbiology, Atlanta, Georgia, March 1-6, 1987.
- Bast, C. B., R. J. M. Fry, and R. J. Preston. Chromosomal and molecular changes associated with fibrosarcoma development in the mouse. 79th Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana, May 25-28, 1988.
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- Bast, Cheryl Barbati, R. Julian Preston, and R. J. M. Fry. Analysis of chromosomal alterations during tumor development. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Bhattacharyya, D., A. M. Boulden, R. S. Foote, and S. Mitra. Properties of human  $O^6$ -methylguanine-DNA methyltransferase. American Society of Biological Chemists, Philadelphia, Pennsylvania, June 7-11, 1987.
- Bhattacharyya, D., R. S. Foote, A. M. Boulden, and S. Mitra. Purification and properties of  $O^6$ -methylguanine-DNA methyltransferase from human placenta. UCLA Symposia on Molecular & Cellular Biology, 1988 Mechanisms and Consequences of DNA Damage Processing, Taos, New Mexico, January 24-30, 1988.
- Bhattacharyya, D., K. Tano, R. S. Foote, and S. Mitra. Rapid purification and properties of *E. coli* Ada protein ( $O^6$ -methylguanine-DNA-methyltransferase). 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, Nevada, May 1-5, 1988.
- Biesiot, P. M., S. Y. Wang, and D. M. Skinner. Discrete segments of a complex eukaryotic satellite are dispersed in a crab genome. 27th Annual Meeting of the American Society for Cell Biology, St. Louis, Missouri, November 16-20, 1987.
- Bingham, G. A. Eradication of mouse hepatitis virus from a breeding and an experimental colony. American Association for Laboratory Animal Science, Chicago, Illinois, October 5-10, 1986.
- Bingham, G. A., and E. M. Garrison. Failure of neomycin therapies to eradicate *Citrobacter freundii* var. 4280 and a carrier state in Balb/C Bd mice. 38th Annual Meeting of American Association for Laboratory Animal Science, Denver, Colorado, November 8-12, 1987.

- Black, Brian L. Influence of temperature on degeneration in *Clostridium acetobutylicum*. Second National Conference on Undergraduate Research, Asheville, North Carolina, April 21-23, 1988.
- Bolch, Wesley E., J. E. Turner, H. A. Wright, R. N. Hamm, H. Yoshida, and K. B. Jacobson. The radiation chemistry of glycylglycine: Monte Carlo calculations of product yields. Thirty-Sixth Annual Meeting of the Radiation Research Society, Philadelphia, Pennsylvania, April 16-21, 1988.
- Bunick, G. J., J. B. Hayter, E. C. Uberbacher, and G. D. Wignall. Oriented scatter from biomolecules using the NCSASR shear cell. Biophysical Society, New Orleans, Louisiana, February 22-26, 1987.
- Cacheiro, N. L. A., K. T. Cain, C. V. Cornett, and J. C. Rutledge. Cytogenetic study of abnormal mouse embryos sired by carriers of balanced reciprocal translocations. Environmental Mutagen Society-Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- Cadilla, C. L., L. H. Cacheiro, and D. E. Olins. Construction of a genomic expression library of macronuclear DNA from *Euplotes eurystomus* and screening for macronuclear protein genes. American Society for Cell Biology, Washington, D.C., December 7-11, 1986.
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- Ch'ang, Lan-Yang, Fred E. Myer, D. M. Yang, C. K. Koh, and W. K. Yang. Promoter and enhancer elements in the long terminal repeats (LTRs) of endogenous murine leukemia virus (MuLV)-related proviral genes. RNA Tumor Virus, Cold Spring Harbor, New York, May 19-24, 1987.
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- Consler, Thomas G., Edward C. Uberacker, Gerard J. Bunick, Michael N. Liebman, and James C. Lee. Effects of ligands on the domain-domain interactions in pyruvate kinase. Biophysical Society Meeting, New Orleans, Louisiana, February 22-26, 1987.
- Cook, John S. Turnover and recycling of cell surface membrane proteins. 9th Conference on Macromolecular Synthesis, Hamburg-Blankenese, FRG, May 24-28, 1987.
- Cosma, G. N., R. Jamasbi, and A. C. Marchok. Benzo(a)pyrene (BAP)- and formaldehyde (HCHO)-induced DNA damage and repair in proliferating primary rat tracheal epithelial cells. American Association for Cancer Research, Atlanta, Georgia, May 20-23, 1987.
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- Crow, Weldon, and Howard Adler. Spontaneous, inheritable changes in *Clostridium acetobutylicum*. Kentucky/Tennessee Branch, American Society for Microbiology, Gatlinburg, Tennessee, November 13-15, 1986.
- Cummings, Robert B. Water chlorination: Risks, benefits, decisions, and the path to the future. Sixth Conference on Water Chlorination: Environmental Impact and Health Effects, Oak Ridge, Tennessee, May 3-8, 1987.
- Dawson, W. D., and J. S. Cook. Biphasic stimulation of A-system amino acid transport by TPA in confluent renal epithelial cells, LLC-PK<sub>1</sub>. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, Nevada, May 1-5, 1988.
- Dawson, W. D., and J. S. Cook. Protein synthesis and the stimulation of A-system amino acid transport by TPA in cultured renal epithelial cells (LLC-PK<sub>1</sub>). Federation of American Societies for Experimental Biology (American Physiological Society), Washington, D.C., March 29-April 3, 1987.
- Dawson, W. D., and J. S. Cook. Retrieval of protein kinase C from a cytosolic fraction of LLC-PK<sub>1</sub> cells by TPA-treated cell membranes and synthetic liposomes. American Society for Cell Biology, Washington, D.C., December 7-11, 1986.
- D'Surney, S. J., and R. A. Popp. Changes in alpha globin gene expression during development of the mouse. 15th Molecular & Biochemical Genetics Workshop, St. Croix, U.S. Virgin Islands, November 3-7, 1987.
- Dunn, W. C., K. Tano, R. S. Foote, G. J. Horesovsky, R. J. Preston, and S. Mitra. Transfer of human O<sup>6</sup>-methylguanine-DNA methyltransferase gene to Chinese hamster ovary cells. UCLA Symposia on Molecular & Cellular Biology, 1988 Mechanisms and Consequences of DNA Damage Processing, Taos, New Mexico, January 24-30, 1988.
- Dunn, W. C., K. Tano, G. J. Horesovsky, D. Bhattacharyya, R. S. Foote, R. J. Preston, and S. Mitra. Human DNA-mediated transfer of O<sup>6</sup>-methylguanine-DNA methyltransferase activity to Mex<sup>-</sup> Chinese hamster ovary cells. 79th Annual Meeting of the American Association for Cancer Research, New Orleans, Louisiana, May 25-28, 1988.
- Engler, David A., Rise K. Matsunami, Stephen R. Campion, Robert S. Foote, Richard J. Mural, Frank W. Larimer, Audrey L. Stevens, and Salil K. Niyogi. Cloning of human epidermal growth factor as a bacterial secretory protein, its properties and mutagenesis. American Society of Biological Chemists, Philadelphia, Pennsylvania, June 7-11, 1987.

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- Epler, J. L., and J. D. Regan. Repair of DNA damaged by activated metabolites of azabenz(a)pyrene in human cells. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Francis, Andrew A., and James D. Regan. Temperature and spectral studies on a UV-induced photosensitive lesion in the DNA of human cells. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Fry, R. J. M. High-LET radiation and the space traveler. Workshop on Risks from Radium and Thorotrast, Bethesda, Maryland, October 3-5, 1988.
- Fry, R. J. M. Radiation effects in space. 8th International Congress of Radiation Research, Edinburgh, Scotland, July 19-24, 1987.
- Fry, R. J. M. Radiation effects in space. Third Workshop on Heavy Charged Particles in Biology and Medicine, GSI, Darmstadt, Germany, July 13-15, 1987.
- Fry, R. J. M. and B. A. Carnes. Age, sex and other factors in radiation carcinogenesis. L. H. Gray Conference, Oxford, England, September 11-15, 1988.
- Fry, R. J. M., R. D. Ley, and L. L. Triplett. Ultraviolet radiation-induced skin cancer. American Society for Photobiology, Colorado Springs, Colorado, March 13-17, 1988.
- Fujimura, R. K. Characterization of T5 DNA polymerase gene. 1987 Evergreen International T4 Meeting, The Evergreen State College, Olympia, Washington, August 1-6, 1987.
- Fujimura, R. K. Characterization of T5 DNA polymerase gene. Invited research seminar to be presented to the scientific staff at Bethesda Research Laboratories (BRL), Gaithersburg, Maryland, November 6-7, 1987.
- Fujimura, Robert K. Research and development in biotechnology: Japanese approach. Online International Organization Meeting on Biotechnology, Pinner, Middlesex, United Kingdom, May 10-12, 1988.

- Gallager, J. F., R. Griesemer, D. M. Popp, and R. A. Popp. Correlation of retroviral integrations and immunocompetence in the lymphatic tissues of B10.F mice. 15th Molecular & Biochemical Genetics Workshop, St. Croix, U.S. Virgin Islands, November 3-7, 1987.
- Gallager, J., R. Griesemer, and D. Popp. Histological study of a murine model of maternally transmitted viremia. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, Nevada, May 1-5, 1988.
- Generoso, W. M. Exposure of mouse zygotes to chemical mutagens: A source of congenital anomalies. Annual Meeting of Scientists Involved in the Office of Health and Environmental Research (OHER) Supported Chemical Toxicology Oriented Research, Monterey, California, June 24-26, 1987.
- Generoso, W. M. Exposure of mouse zygotes to chemical mutagens: A source of congenital anomalies. (Extended abstract.) Annual Meeting of Scientists Involved in the Office of Health and Environmental Research (OHER) Supported Chemical Toxicology Oriented Research, Monterey, California, June 24-26, 1987.
- Generoso, W. M. Zygote-derived developmental anomalies, a new endpoint of mutagenesis. Twenty-Seventh Hanford Life Sciences Symposium "Multilevel Health Effects Research: From Molecular to Man," Richland, Washington, October 18-20, 1988.
- Ghiron, Camillo A., Maurice R. Eftink, Michael A. Porter, and Fred C. Hartman. Fluorescence studies of phosphoribulokinase, a light-regulated, chloroplastic enzyme. Annual Meeting of the Biophysical Society, Phoenix, Arizona, February 23-March 3, 1988.
- Godfrey, V. L., R. A. Griesemer, and L. B. Russell. Clinical and histopathologic characteristics of the *Scrufty* mutant mouse. The 39th Annual Meeting of the American College of Veterinary Pathologists, Kansas City, Missouri, October 31-November 4, 1988.
- Greer, G. A., and R. J. Preston. Characterization of an ara-C resistant X-ray sensitive CHO mutant. Environmental Mutagen Society-Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- Griesemer, R. A. Carcinogenicity protocols. International Symposium on Inhalation Toxicology, Hannover, Federal Republic of Germany, March 23-27, 1987.
- Hartman, Fred C., Robert S. Foote, Frank W. Larimer, Eva H. Lee, Richard Machanoff, Sylvia Milanez, Sankar Mitra, Richard J. Mural, Salil K. Niyogi, Thomas S. Soper, and Claude D. Stringer. Function of active-site lysines of ribulose biphosphate carboxylase/oxygenase. Oji International Seminar on "New Aspects of Plant Cell Biology and Molecular Biology, Kashiko-Jima, Japan, October 20-24, 1986.

- Hauser, Melinda, and John S. Cook. Uptake of putrescine into cultured renal epithelial cells, LLC-PK<sub>1</sub>. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, Nevada, May 1-5, 1988.
- Hix, Cathy L., Andrew A. Francis, William L. Carrier, and James D. Regan. A photobiological evaluation of tanning beds. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Hook, G. J., and R. J. Preston. The effect of incorporation of base analogues on the number and type of X-ray induced chromosome aberrations. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Hook, G. J., and R. J. Preston. Restriction endonucleases-induced chromosome aberrations in human lymphoblastoid cells. Environmental Mutagen Society - Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- Horesovsky, G. J., W. C. Dunn, S. Mitra, and R. J. Preston. The induction of chromosome aberrations and mutations in CNU-resistant Chinese hamster ovary cells. Environmental Mutagen Society - Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- Horesovsky, G. J., and R. J. Preston. Restriction enzyme-induced mutations in CHO cells. Environmental Mutagen Society, San Francisco, California, April 8-11, 1987.
- Jacobson, K. Bruce, M. W. England, S. E. Holt, D. L. Nida, and H. J. Gill. Mechanism of cadmium resistance in *Drosophila*. UCLA Symposium "Metal Ion Homeostasis: Molecular Biology and Chemistry," Frisco, Colorado, April 10-16, 1988.
- Jacobson, K. Bruce and R. E. Manos. Effects of 6-acetylhomopterin and sepiapterin on GTP cyclohydrolase from mouse liver and *Drosophila* head. Annual International Pteridine Workshop "Pteridines and Related Biogenic Amines," Snowbird, Utah, March 13-18, 1988.
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- Jamison, C. Scott, and Howard I. Adler. Mutations in several *Escherichia coli* chromosomal regions affect sensitivity to oxygen. Kentucky/Tennessee Branch, American Society for Microbiology, Gatlinburg, Tennessee, November 13-15, 1986.
- Jamison, C. Scott, and Howard I. Adler. Mutations in six *Escherichia coli* chromosomal regions effect sensitivity to oxygen. American Society for Microbiology, Atlanta, Georgia, March 1-6, 1987.

- Jenkins, N. A., M. C. Strobel, P. K. Seperack, D. M. Kingsley, K. J. Moore, L. B. Russell, and N. G. Copeland. A retroviral insertion in the dilute locus provides molecular access to this region of mouse chromosome 9. Alexander Hollaender Fortieth Anniversary Gatlinburg Symposium on Transposable Elements in Mutagenesis and Regulation of Gene Expression, Gatlinburg, TN, April 11-14, 1988.
- Johnson, D. K. and E. M. Rinchik. Mapping of random DNA clones to two radiation-induced deletion complexes in mouse chromosome 7. Sixth International Workshop on the Molecular Genetics of the Mouse, Cambridge, England, July 3-8, 1988.
- Katz, D. Sue, Abraham W. Hsie, Mark C. Jernigan, and Robert L. Schenley. The effect of anaerobic conditions on CHO cell killing and 6-TG mutagenesis by neutrons. Radiation Research Society - 36th Annual Meeting, Philadelphia, Pennsylvania, April 18-21, 1988.
- Kennel, Stephen J., Rita Falcioni, and Ada Sacchi. Two-site monoclonal antibody assay for human TSP-180. Second Conference on Immunity to Cancer, Williamsburg, Virginia, November 9-11, 1987.
- Kennel, S. J., T. K. Lankford, R. L. Ullrich, and R. J. Jamasbi. Treatment of mice with monoclonal antibodies specific for lung endothelial cells enhances lung tumor colony formation. American Association of Cancer Research, Inc. 79th Annual Meeting, New Orleans, Louisiana, May 25-28, 1988.
- Kenney, F. T., K.-L. Lee, and K. R. Isham. Studies on the mechanism of insulin control of gene expression. 14th International Congress of Biochemistry, Prague, Czechoslovakia, July 10-15, 1988.
- Kohmescher, D. C., and R. J. Preston. Chromosome aberration induction and cell cycle progression in an X-ray sensitive CHO cell. Environmental Mutagen Society - Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- Larimer, Frank W., Richard Machanoff, and Richard J. Mural. High level expression of *Rhodospirillum rubrum* ribulose bisphosphate carboxylase/oxygenase in *Escherichia coli*. International Workshop on Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase: Genes, Proteins, and the Regulation of Activity, University of Arizona, Tucson, Arizona, April 20-25, 1987.
- Larimer, F. W., J. R. Perry, and A. A. Hardigree. Structure and function of *REV1* gene of yeast. Yeast Genetics and Molecular Biology Meeting, San Francisco, California, June 16-21, 1987.

- Larimer, Frank W., Thomas S. Soper, Richard J. Mural, Richard Machanoff, and Fred C. Hartman. Essentiality of Glu-48 of ribulose biphosphate carboxylase/oxygenase as demonstrated by site-directed mutagenesis. First Symposium of the Protein Society, San Diego, California, August 9-13, 1987.
- Lee, Eva H., and Fred C. Hartman. Catalysis of proton exchange between ribulose biphosphate (RuBP) and solvent by site-directed mutants of RuBP carboxylase/oxygenase deficient in overall carboxylase activity. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, Nevada, May 1-5, 1988.
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- Lewis, B. A., and J. S. Cook. NMR studies of phorbol ester and diglyceride effects on phospholipid structure. Biophysical Society, New Orleans, Louisiana, February 22-26, 1987.
- Machanoff, R., F. W. Larimer, R. J. Mural, S. K. Niyogi, R. S. Foote, T. S. Soper, S. Mitra, and F. C. Hartman. Protein engineering of ribulose biphosphate carboxylase. Kentucky/Tennessee Branch, American Society for Microbiology, Gatlinburg, Tennessee, November 13-15, 1986.
- Mansfield, B. K., R. C. Mann, and J. K. Selkirk. Expression of cytoplasmic proteins in Friend erythroleukemia cells chemically induced to undergo terminal erythroid differentiation: Two-dimensional gel electrophoresis as a tool for determining protein modulations. American Association for Cancer Research, Atlanta, Georgia, May 20-23, 1987.

- Marchok, A. C., and G. N. Cosma. Quantitation of tumor-initiation sites induced by benzo(a)pyrene (BAP) plus formaldehyde (HCHO) using the open-ended rat tracheal implant (OETI)-cell culture model. American Association for Cancer Research, Atlanta, Georgia, May 20-23, 1987.
- Marchok, A. C., G. S. Fleming and G. N. Cosma. Model compounds and complex mixtures in the open-ended rat tracheal implant model: Effects and metabolism. Second Annual DOE Contractors Meeting on Health and Environmental Effects of Complex Chemical Mixtures, Delavan, Wisconsin, June 16-18, 1986.
- Margaretten, N. C., and H. P. Witschi. Effects of hyperoxia on tumor cell antioxidant defenses and cell kinetics in vivo. Society of Toxicology, Washington, D.C., February 23-27, 1987.
- Margaretten, N. C., and H. P. Witschi. Hyperoxia reduces experimental lung metastasis from sensitive cell lines. Society of Toxicology, Washington, D.C., February 23-27, 1987.
- Margaretten, N. C., and H. P. Witschi. Induction of oxygen tolerance in mice. Society of Toxicology, Washington, D.C., February 23-27, 1987.
- Mazur, Peter. Basic concepts in freezing. Workshop on Sperm Cryopreservation, National Institutes of Health, Bethesda, Maryland, January 18-19, 1988.
- Mazur, P. Does the unfrozen water fraction play a role in freezing injury, or is water activity the main culprit? 25th Annual Meeting, Society for Cryobiology, Aachen, W. Germany, July 11-15, 1988.
- Mazur, Peter. Stopping biological time: The freezing of living cells. Office of Health and Environmental Research, U.S. Department of Energy, Washington, D.C., February 5, 1987.
- Mazur, Peter. Stopping biological time: The freezing of living cells. Plenary Session, Vth World Congress on In Vitro Fertilization and Embryo Transfer, Norfolk, Virginia, April 9, 1987.
- Mazur, P. and K. W. Cole. Contact between shrunken red cells as a factor in freezing injury. 25th Annual Meeting, Society for Cryobiology, Aachen, W. Germany, July 11-15, 1988.
- Mazur, P. and K. W. Cole. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. 25th Annual Meeting, Society for Cryobiology, Aachen, W. Germany, July 11-15, 1988.

- Mazur, P., U. Schneider, K. B. Jacobson, and A. P. Mahowald. Chilling injury in intact *Drosophila* eggs at various stages of embryonic development between 0° and -25°C in the absence of ice formation. 25th Annual Meeting, Society for Cryobiology, Aachen, W. Germany, July 11-15, 1988.
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- McKarns, S. C., K. N. Nikbakht, W. K. Yang, and R. J. Preston. Correlation of metacentric indicator chromosomes and oncogene activation with neoplastic progression *in vitro*. Environmental Mutagen Society - Nineteenth Annual Meeting, Charleston, South Carolina, March 27-31, 1988.
- McKarns, S. C., R. L. Ullrich, and R. J. Preston. Chromosome analysis of murine mammary clonal cell lines; possible role of DMs, HSRs, and metacentric chromosomes in neoplastic progression. 2nd International Workshop on Chromosomes in Solid Tumors, Tucson, Arizona, January 18-20, 1987.
- Mendel, Jane E., Edward G. Bernstein, Liane B. Russell, Peter A. Lalley, and David E. Housman. Genomic cloning of mouse lactate dehydrogenase A: Use of deletion mutants to distinguish an expressed gene from its pseudogene counterparts. 14th Molecular and Biochemical Genetics Workshop, Bar Harbor, Maine, August 26-30, 1986.
- Milanez, Sylvia, and Richard J. Mural. Sequence of the gene for phosphoribulokinase (PRK) from spinach. UCLA Symposia on Molecular Basis of Plant Development, Steamboat, Colorado, March 22-April 2, 1988.
- Mitchell, D. L., L. H. Thompson, J. D. Regan, S. A. Stewart, W. L. Carrier, and R. S. Nairn. CHO mutant UV61 removes (6-4) photoproducts but not cyclobutane dimers. UCLA Symposia on Molecular & Cellular Biology, 1988, Mechanisms and Consequences of DNA Damage Processing, Taos, New Mexico, January 24-30, 1988.
- Mural, Richard J., Robert S. Foote, Frank W. Larimer, Eva H. Lee, Richard Machanoff, Sankar Mitra, Salil K. Niyogi, Thomas S. Soper, and Fred C. Hartman. Examination of the role of active site residues of ribulose biphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* by site-directed mutagenesis. Protein Engineering '87, Nuneham Park, Nuneham Courtenay, Oxon, United Kingdom, April 5-8, 1987.

- Mykles, D. L., and D. M. Skinner. Four  $\text{Ca}^{2+}$ -dependent proteinases in crustacean muscles: possible regulation by activators and inhibitors. American Society for Cell Biology, Washington, D.C., December 7-11, 1986.
- Niemann, S. L., and R. A. Popp. Ethylene oxide induces stage-specific germ cell damage in SEC and C57BL/6 mice. Environmental Mutagen Society Meeting, Charleston, South Carolina, March 27-31, 1988.
- Niemann, S. L., and R. A. Popp. Genetic basis for differential sensitivity to ethylene oxide in strains of mice. 15th Molecular & Biochemical Genetics Workshop, St. Croix, U.S. Virgin Islands, November 3-7, 1987.
- Nikbakht, K. N., D. M. Yang, L.-Y. Ch'ang, and W. K. Yang. Functional characterization of a mouse endogenous MuLV-related proviral DNA clone stably transfected into a human fibrosarcoma cell line. RNA Tumor Virus, Cold Spring Harbor, New York, May 19-24, 1987.
- O'Brien, J. J., and D. Skinner. Degradation of exoskeletal proteins by crab (*Gecarcinus lateralis*) proteinase. American Society of Zoologists, New Orleans, Louisiana, December 27-30, 1987.
- O'Brien, J. J., and D. M. Skinner. Proteinases involved in cyclic degradation of crustacean exoskeleton. American Society of Zoologists, Nashville, Tennessee, December 27-30, 1986.
- Otten, James A. Sampling for bioaerosols in the indoor environment. American Biological Safety Association, Meadowlands, (Secacus, New Jersey), October 18-21, 1987.
- Otten, J. A. Sampling viable bioaerosols. American Chemical Society-"Exchange of Contaminants Across the Atmosphere/Surface Interface in Agriculture," Los Angeles, California, Fall (September), 1988.
- Otten, J. A., H. A. Burge, M. A. Chatigny, J. C. Feeley, K. Kreiss, P. R. Morey, K. Peterson, and J. J. Tulis. Assessing bioaerosols in the indoor environment: Guidelines. Annual Meeting of the American Association for Aerosol Research, Chapel Hill, North Carolina, October 10-13, 1988.
- Paulson, C. R., and D. M. Skinner. *In vitro* effects of 20-hydroxyecdysone and juvenile hormone on crab tissue. American Society of Zoologist, New Orleans, Louisiana, December 27-30, 1987.
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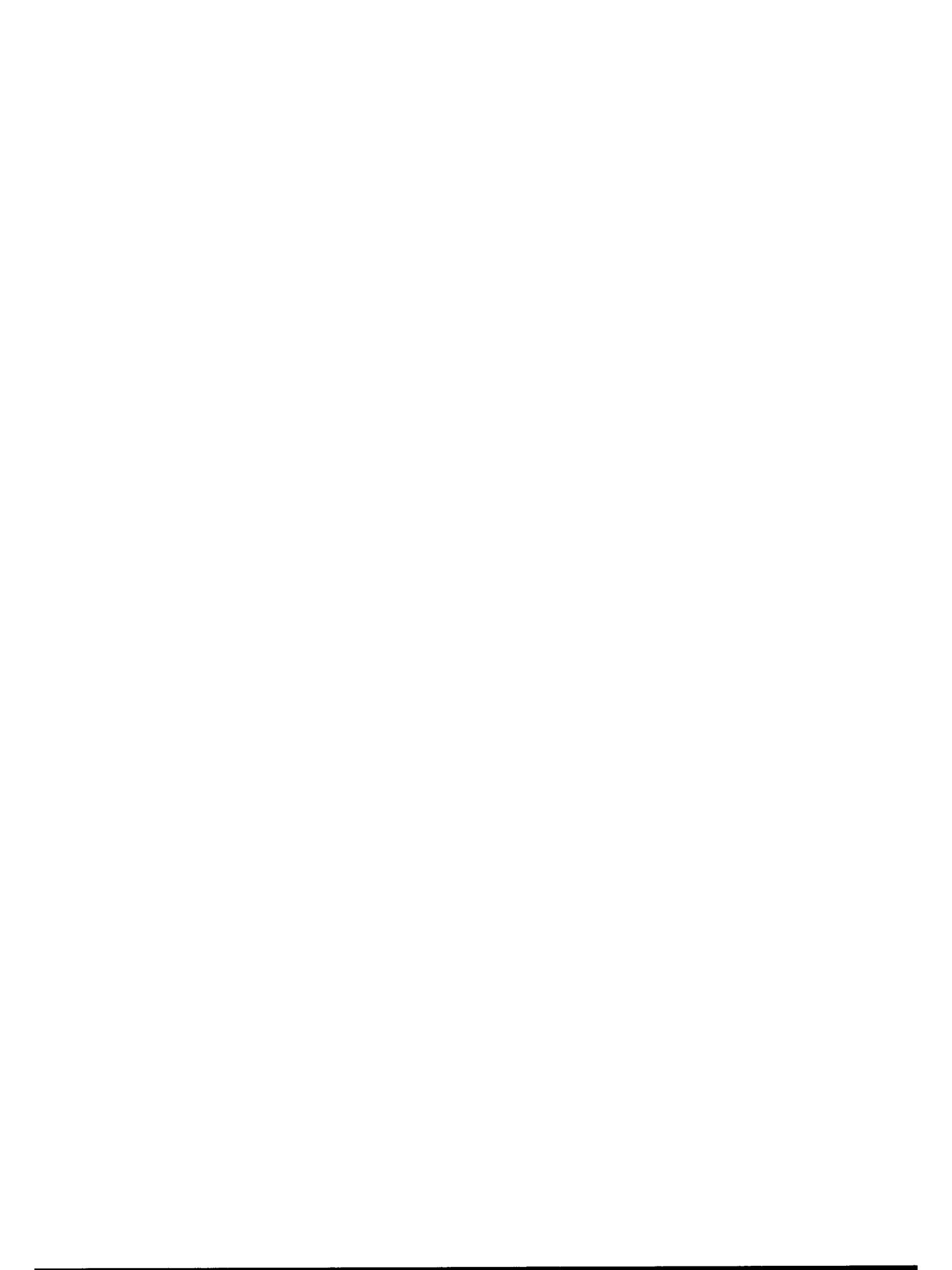
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## Financial Summary and Personnel Distribution

### Total Biology Division

FY 1988

Funding Source	Funding in Thousands	Percent of Total Budget	Scientific/ Technical Person-Years <sup>1</sup>
Department of Energy	10,833	78.5	65.0
National Institute of Environmental Health Sciences	1,278	9.2	9.0
National Cancer Institute	889	6.4	6.5
University of Tennessee	248	1.8	1.7
National Institute on Ageing	175	1.3	.9
National Institute of General Medical Sciences	137	1.0	.1
National Heart, Lung and Blood Institute	136	1.0	.9
Department of Agriculture	66	.5	.2
Veterans Administration	28	.2	.2
Miscellaneous	11	.1	.1
	<hr/> 13,800	<hr/> 100.0	<hr/> 84.6

<sup>1</sup>Does not include ~64 person years: 51 PY Distributed - Administration & clerical, animal caretakers, histology, and kitchen; 13 PY supported by other divisions and ORNL seed money.



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