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APR 30 1979

Mr. Joe Federline
Blair Building, Room B-16
Carcinogenesis Contracts Section
Research Contracts Branch
National Cancer Institute
National Institute of Health
Bethesda, Maryland 20014

Dear Mr. Federline:

ORNL PROPOSAL ENTITLED "GENETIC ANALYSIS OF DNA REPAIR IN MAN WITH CELL HYBRIDS," SUPPLEMENT TO CHEMICAL CARCINOGENESIS PROGRAM (INTERAGENCY AGREEMENT DOE NO. 40-5-63, NCI NO. Y01-CP 50200)

Enclosed for your consideration are twenty-five (25) copies of a summary document, fifteen (15) copies of the technical proposal, and six (6) copies of the business management cost proposal for the subject supplement to the on-going interagency agreement. The proposal is submitted on the basis of discussions between Peter A. Lalley and R. J. M. Fry, ORNL, and David Longfellow, NCI Project Director. Proposed is a three-year research effort with NCI funding in the amount of $265,300, of which $82,000 will be required for the initial twelve-month period of performance. Peter A. Lalley and James D. Regan will be co-principal investigators for ORNL.

We will be pleased to authorize ORNL to conduct the subject research upon receipt of a NCI fund authorization in the amount of $82,000 to support the first year's effort.

Sincerely,

Original Signed by
William R. Bibb

William R. Bibb, Director
Research Division

ER-13:WRB

Enclosures:
Summary of Proposal (25)
Technical Proposal (15)
Business/Cost Proposal (6)

cc w/encls:
C. W. Hill

cc w/o encls:
Peter A. Lalley, ORNL
Herman Postma, ORNL

REPOSITORY Oak Ridge Operations Records Holding Area
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40-5-63 NCI # Y01CP50200
FOLDER NIH April 13, 1979

CONCURRENCES

RTG. SYMBOL	INITIALS/SIG.	DATE
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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION
NUCLEAR DIVISION

POST OFFICE BOX X
OAK RIDGE, TENNESSEE 37830

March 12, 1979

OFFICE OF THE DIRECTOR

Approved by Madden 4/2/79

Department of Energy
Oak Ridge Operations
Attention: Mr. J. A. Lenhard, Assistant Manager
for Energy Research and Development
Post Office Box E
Oak Ridge, Tennessee 37830

Gentlemen:

Supplement to NCI Interagency Agreement 40-5-63
"Genetic Analysis of DNA Repair in Man with Cell Hybrids"

Enclosed for your review and approval is a supplemental proposal being submitted to the National Cancer Institute based upon discussions with Peter A. Lalley and R. J. M. Fry, ORNL, and David Longfellow, NCI Project Director. This proposal is a supplement to existing DOE Agreement 40-5-63, NCI Y01 CP 50200, and requests a three-year effort beginning July 1, 1979. The initial twelve-month period will require funding in the amount of $82,000.

The following information is provided to assist in your review and approval of this supplement:

a. This research work is described in the approved ORNL Institutional Plan on page 52, "Work for Others (Excluding NRC)." The personnel required for this effort are included in the direct FTE's for DHEW in the "Summary of Resources" on page 53.

b. The principal investigators for this research will be Peter A. Lalley (20%) and James D. Regan (10%). Dr. Lalley's time will reduce his current efforts on his DOE project, "Genetic Basis for Mutagenesis/Carcinogenesis," and Dr. Regan's time will be taken from existing NCI efforts.

c. Existing space and resources are available to conduct this research.

d. This research will serve the DOE-related objectives in Activity GK 01 02 02 2, "Health Effects Research in Biological Systems - Mutagenesis." Based upon this programmatic benefit, it is requested that this research be conducted on an actual cost basis, depreciation and DOE added cost factor waived.

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After your approval and approval of the Office of Health and Environmental Research, please forward 25 copies of the summary document, 15 copies of the technical proposal, and six copies of the business management cost proposal to Joe Federline, Blair Building, Room B-16, Carcinogenesis Contracts Section, Research Contracts Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014.

If there are any questions relating to this proposal, please contact C. R. Richmond, ext. 4-4332.

Sincerely yours,

Herman Postma
Director

HP:VBI:kh

Enclosure

cc: R. J. M. Fry
R. F. Hibbs
P. A. Lalley
J. N. Maddox, DOE-GTN
W. R. Ragland
C. R. Richmond
J. B. Storer
File - RC

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I. SUMMARY DOCUMENT
For
UNSOLICITED PROPOSAL

Date of Submission:	December 6, 1978
Title of Proposed Project:	Genetic Analysis of DNA Repair in Man with Cell Hybrids
Co-Principle Investigators:	Dr. Peter A. Lalley Dr. James D. Regan
Institution:	Biology Division P. O. Box Y Oak Ridge National Laboratory Oak Ridge, Tennessee 37830
Proposed Level of Effort:	Peter A. Lalley: 20% of time James D. Regan : 10% of time
Total Hours of Other Support Personnel:	52 hrs/week

This proposal ( ) does, (X) does not involve recombinant DNA research

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TECHNICAL SUMMARY OF PROPOSAL

Rationale

A large amount of experimental evidence exists which indicates that damage to DNA is mutagenic and that physical and chemical mutagens can be carcinogens (1,2). It is evident, therefore, that the mechamisms by which cells repair damage to their DNA play a crucial role in carcinogenesis such that inefficient or inaccurate repair should correlate with increased rates of carcinogenesis.

A unique human disease which demonstrates a remarkable association between damage to DNA and carcinogenesis is the recessively inherited skin disease xeroderma pigmentosum (XP) in which the DNA repair mechanism has been shown to be defective (3). The major clinical feature of this disease is a high incidence of sunlight-induced skin cancers of all cell types (4,5). It has been demonstrated that the biochemical lesion in XP cells is a defect in excision repair of UV damage of DNA and it is postulated to be at the level of the endonucleolytic incision of UV-irradiated DNA (6,7). In addition, cells derived from XP patients show an exceptional susceptibility to mutagenic agents (8). Thus, XP patients provide a direct link between defective DNA excision repair, induction of mutations, and induction of cancer. These observations establish the primary importance of DNA repair mechanisms in preserving and monitoring the genetic integrity ofcellular DNA, and correcting DNA damage induced by carcinogens and mutagens.

While the molecular biology of this repair system has been advanced during the past ten years, the genetic structure of excision repair of UV-induced damage has not been well characterized. The genetic heterogeneity of XP(six complementation groups) raises questions since the supposed biochemical lesion is thought to be the UV endonuclease which initiates excision of UV-induced pyrimidine dimers (5,6,7,9).

a) Is the genetic defect responsible for XP located at a single gene locus which can be assigned to a single chromosome, or are mutations affecting several gene loci involved?

b) Does the genetic defect associated with XP constitute a mutation of a structural gene or genes, or is it the result of a mutation at control or regulatory gene loci?

c) Does the fusion of XP cells itself alter gene expression in an epigenetic way such that the number of complementation groups does not indicate the number of gene loci involved?

Thus, the available information on XP does not fully indicate the genetic or molecular origin of the disorder.

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OBJECTIVES AND GENERAL APPROACHES

It is imperative to distinguish among the possibilities listed above if the functional relationships between DNA repair, mutagenesis and carcinogenesis are to be fully understood, and XP is to serve as a model system for the study of radiation and chemically induced carcinogenesis.

Therefore, the primary objectives of this project will be to (a) genetically dissect the DNA excision repair system in man; (b) identify the number and kinds of genes required for DNA repair; (c) assign these genes to specific human chromosomes; (d) determine the nature of the genetic defect in XP; (e) map the genes responsible for XP on a specific human chromosome(s).

Our technical capacities to pursue this study reside in the ability to generate and genetically analyze man x mouse somatic cell hybrids for the presence of each human chromosome and the ability to repair DNA damage, employing several very sensitive, rapid and informative experimental techniques. The assays for DNA repair include (a) bromodeoxyuridine photolysis, (b) radiochromatography and (c) molecular weight analysis which measure two or more steps involved in repair. Proliferating man x mouse somatic cell hybrids are particularly suited for this study. Human cells excise half of the UV light induced pyrimidine dimers in 24 hours, while mouse cells excise only about 3%; therefore, the excision repair seen in man x rodent cells can be differentiated as to human or mouse origin (10). The preferential loss of human chromosomes in proliferating man x mouse hybrids, the interspecific differences between homologous gene products, and the availability of techniques to identify specific human chromosomes have made it possible to determine gene-chromosome assignments (11,12) and to dissect a complex polygenic system by isolating its component parts (13,14,15).

Our methods permit analysis of the individual contributions made by structural genes, processing genes and control genes to the genetic structure of the human repair mechanism. The importance of these studies lies in:

a) The fact that most organisms, including man, possess several complex DNA repair systems and that an elucidation of the genetics of one system will aid in our understanding of the other systems;

b) The demonstrated association between defective DNA repair, cancer-proneness and increased sensitivity to physical and chemical mutagens and carcinogens;

c) The need to determine the number and kind of genes required for excision repair of UV-induced damage in order to understand the interactions of the numerous enzymes required for DNA repair;

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d) The fact that a knowledge of the chromosomal assignment of the genes required for DNA repair and the gene or genes defective in XP will be extremely useful in prenatal diagnosis and genetic counseling.

In order to pursue this project, somatic cell hybrid lines have been generated. Human cells derived from several different individuals with normal DNA repair capacity have been fused to established mouse cell lines using inactivated Sendai virus or polyethylene glycol as fusing agents. Primary hybrid clones have been isolated in the HAT selection system (11). All clones isolated under these conditions preferentially segregate human chromosomes and retain the full complement of mouse chromosomes. These clones are being employed to determine the chromosomal assignments of the gene(s) coding for DNA repair enzymes in man.

Previous repair studies showed that mouse cells have less than 10% of the human excision capacity (10). Thus, it is possible to distinguish the human DNA repair components in the human x mouse somatic cell hybrids. Man x mouse hybrid clones can be analyzed for DNA repair capacity following UV radiation, and the ability to repair UV damage will be compared to the presence or absence of 35 human enzyme markers. Genes coding for these enzyme markers have been assigned to each human chromosome except the Y chromosome (12,16). Concordant segregation of the ability to repair UV damage with specific gene markers will determine the gene-gene linkage relationship, and by implication, the chromosomal assignment(s) of the gene(s) involved in DNA repair in man. The three goals of this program are: (a) to determine the amount of DNA repair in hybrid cells; (b) to determine the number of genes required to carry out this process; and (c) to assign these genes to specific human chromosomes. Our techniques permit relation of the amount of DNA repair to gene dosage. Many of the hybrids will be haploid for human chromosomes. Other hybrids will retain both homologues and be functionally diploid, having two copies of the genes located on that chromosome pair. Other hybrids will have lost both homologues and be deficient for the genes located on that chromosome pair. If the amount of DNA repair is gene dosage dependent, then three classes of hybrids should be found: (a) hybrids negative for human DNA repair; (b) hybrids possessing 50-60% of the normal human excision capacity; (c) hybrids possessing 100% of the normal human excision capacity. The negative hybrids would be analogous to XP patients, hybrids in the second class would be analogous to heterozygotes for DNA repair, and hybrids in the third class would be analogous to individuals normal for DNA repair. If DNA repair is not gene dosage dependent, then only negative hybrids and hybrids expressing 100% of normal human excision capacity should be found. Differentiation between these two possibilities will have important implications for determining individuals heterozygous for xeroderma pigmentosum and for prenatal diagnosis of this genetic defect.

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An important question that must be answered is whether or not the mouse input cells can complement defective DNA repair in human cells. Therefore, man x mouse somatic cell hybrids will be generated employing human cells derived from at least five different XP complementation groups. The existence of six complementation groups (9) may suggest that there are multiple subunits for human UV endonuclease, or that the process of cell hybridization alters gene expression such that complementation in vitro is not a simple indicator of the number of different gene loci involved. If human DNA repair is complemented in XP x mouse hybrids, then the nature of this complementation can be determined by generating XP x mouse hybrids which segregate mouse chromosomes. Procedures have been described for the generation and analysis of hybrids which segregate mouse chromosomes (17,18,19). If the mouse is supplying a specific gene product, the loss of human DNA repair capacity should correlate with the loss of a specific mouse chromosome. If complementation is an epigenetic effect, then no such correlation will be seen. These studies will help to elucidate the precise genetic defect in xeroderma pigmentosum.

The knowledge of the genetic structure of excision repair in man will provide new insights and understanding of how damage to DNA by physical and chemical agents can be carcinogenic.

We believe that this approach to the genetic dissection of DNA repair mechanisms with cell hybrids can be uniquely carried out in our combined laboratories.

The complex experimental interactions necessary to a genetic analysis of human DNA repair require that a somatic cell genetics laboratory and a DNA repair laboratory work simultaneously on the same passage of hybrid cells. This is due to the fact that proliferating human x mouse cell hybrids may continue to lose human chromosomes as they are passaged for extended periods or after they have been removed from storage in liquid nitrogen. If the genetic analysis is performed on one passage of a primary hybrid clone and the DNA repair assays are performed on another passage of that same clone, it will not be legitimate to compare the data since the human chromosome complement in the hybrid cell may have changed.

Dr. Lalley's group performs cytogenetic and enzymatic analysis in the hybrid cells while the same cells are undergoing analyses for DNA repair in Dr. Regan's laboratory. This allows for a rapid and accurate correlation of data which is absolutely necessary for successfully performing this work.

There are several excellent laboratories throughout the country devoted to DNA repair studies or to cell hybrid studies. However, we know of no other laboratory situation where genetic analysis of hybrids and DNA repair studies on the same passage of hybrids is being conducted in such a closely-knit fashion.

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SUMMARY DOCUMENT

3) DIRECT COST

	Direct Labor	Equipment	Other Costs	Total
First Year	$33,800	$ -	$13,500	$47,300
Second Year	36,200	-	14,800	51,000
Third Year	38,700	-	16,100	54,800

4) FINANCIAL SUPPORT

Agency	Title
DOE	Genetic Basis of Mutagenesis/Carcinogenesis-FY 1979 $120,000 (Lalley)
DOE	Medical and Molecular Genetics-FY 1979 - $234,000 (Regan)
NASA	UV-Induced DNA Damage and Repair in Cultured Cells, T-1819E, FY 1979 - $24,000 (Regan)

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II. TECHNICAL PROPOSAL
For
UNSOLICITED PROPOSAL

Date of Submission:	December 6, 1978
Title of Proposed Project:	Genetic Analysis of DNA Repair in Man with Cell Hybrids
Co-Principle Investigators:	Dr. Peter A. Lalley Dr. James D. Regan
Institution:	Biology Division P. O. Box Y Oak Ridge National Laboratory Oak Ridge, Tennessee 37830
Proposed Level of Effort:	Peter A. Lalley: 20% of time James D. Regan : 10% of time
Total Hours of Other Support Personnel :	52 hrs/week

This proposal ( ) does, (X) does not involve recombinant DNA research

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TABLE OF CONTENTS

Introduction..... 1

Objectives and Scope..... 3

Background..... 5

Experimental..... 10

1. Cell Culture..... 10

2. Cell Hybridization..... 11

3. Detection of DNA Single-Strand Breaks on Alkaline Sucrose Gradients..... 11

4. The dBrU Photolysis Assay..... 12

5. Direct Measurement of Thymine-containing Dimers in UV-irradiated Cellular DNA..... 13

6. Enzyme Analysis..... 13

7. Karyotype Analysis..... 13

8. Prereplication Repair in Man x Mouse Somatic Cell Hybrids Following UV Radiation..... 13

9. Formation of Man x Mouse Somatic Cell Hybrids Utilizing Different XP Cells as the Human Input Cells..... 15

References..... 16

Direct Cost Budget..... 18

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INTRODUCTION

A unique human disease which demonstrates a direct linkage between environmental and genetic factors and carcinogenesis is the skin disease xeroderma pigmentosum (XP) in which the DNA repair mechanism has been shown to be defective (1,2). The major clinical feature of this disease is a high incidence of sunlight-induced skin cancers of all cell types (3,4). In addition, cells derived from XP patients show an exceptional susceptibility to mutations induced by UV radiation or chemicals (5). Thus XP patients provide a direct link between defective DNA repair, induction of mutations, and induction of cancer. These observations establish the primary importance of DNA repair mechanisms in preserving and monitoring the genetic integrity of cellular DNA, and correcting DNA damage induced by carcinogens and mutagens.

Genetic studies utilizing the fusion of XP cells from unrelated patients have indicated the existence of at least six different complementation groups, designated complementation group A through F, in the excision deficient class of XP patients (6). This genetic heterogeneity of XP is paradoxical since the biochemical lesion has been suggested to be the UV endonuclease which initiates excision of UV-induced pyrimidine dimers from DNA (7). Several hypotheses have been advanced to explain these data including:

a) the speculation that UV endonuclease is composed of several protein subunits, each coded for by a different gene;

b) the groups represent genes coding for enzymes in a common repair pathway, e.g. endonuclease, exonuclease, "editing" enzyme, repair polymerase, and polynucleotide ligase.

c) the groups represent intragenic complementation of different mutations in a single gene coding for a repair endonuclease;

d) the involvement of control or regulatory gene loci;

e) the possibility that cell fusion itself alters gene expression such that the number of complementation groups is not indicative of the number of loci involved. Thus, the available characterization of this excision repair deficient disorder does not fully indicate its genetic or molecular origin.

It is imperative to investigate the genetic structure of DNA repair in man if we are to fully understand the functional relationship between excision repair, mutagenesis and carcinogenesis.

We have developed a project designed to analyze the genetic basis of DNA excision repair in man employing man x mouse somatic cell

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hybrids as our experimental system. We feel this is a very favorable system for the following reasons. Since DNA repair of UV-induced lesions in the mouse is quantitatively different from that seen in human cells, the repair seen in man x mouse somatic cell hybrids can be differentiated as to human or mouse origin (8). The preferential loss of human chromosomes in proliferating hybrids, the interspecific differences between homologous gene products, and the availability of techniques to identify specific human chromosomes make it possible not only to determine gene-chromosome assignments, (9,10) but also to dissect a complex polygenic system by isolating its component parts (11,12,13).

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OBJECTIVES AND SCOPE

The purpose of the proposed research is to investigate the expression and individual genetic components involved in DNA repair in man. Such an elucidation of the genetic basis of DNA repair is fundamental if we are to understand its pivotal role in carcinogenesis. Therefore, the primary objectives of this project will be (a) to genetically dissect the DNA repair systems in man; (b) to identify the number and kinds of genes required for DNA repair; (c) to assign these genes to specific human chromosomes; and (d) to identify and determine the chromosomal assignment(s) of the genetic defect in XP.

Our technical capacities to carry out this study reside in the ability to generate and genetically analyze man x mouse somatic cell hybrids for the presence on each human chromosome, and for the ability to repair DNA damage employing several very sensitive and informative experimental techniques. The assays for DNA repair include (a) bromodeoxyuridine photolysis, (b) radiochromatography and (c) molecular weight analysis. These assays measure three different steps in excision repair. The presence or absence of specific human chromosomes is determined enzymatically by assaying each hybrid clone for the expression of 35 human enzymes which provide gene markers for each human chromosome except the Y, and cytogenetically by identifying specific human chromosomes in the hybrid clones employing standard chromosome banding techniques.

Somatic cell hybrids have been formed between human cells derived from several individuals normal for DNA repair and established mouse cell lines. These hybrids will be assayed for their ability to repair UV damage and for the presence or absence of specific human chromosomes. Somatic cell hybrids will also be formed between XP cells from the various complementation groups and mouse cells. These hybrids will serve as controls in order to determine whether or not the mouse input cells can complement defective DNA repair in human cells.

Repair of UV radiation-induced damage will serve as a model system for the genetic analysis of human repair mechanisms. Following an investigation of this system, we will apply the same techniques to investigate the genetic structure of the mechanisms involved in repairing chemically induced DNA damage. Several lines of evidence indicate that the repair modes by which human cells respond to physical or chemical results may be different (cf. Setlow, R. B. Nature 271: 713-717, 1978). We will investigate this question from a genetic point of view.

The importance of these studies lies in (a) the fact that most organisms, including man, possess several complex DNA repair systems; (b) the demonstrated association between defective DNA repair,

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cancer-proneness, and increased sensitivity to physical and chemical environmental mutagens and carcinogens; (c) the need to elucidate the genetic basis of this polygenic system in order to understand the interactions of the numerous repair enzymes; and (d) the fact that a knowledge of the chromosomal assignment of the genes required for DNA repair and the gene or genes defective in XP will be extremely useful in prenatal diagnosis and genetic counseling.

We believe that this approach to the genetic dissection of DNA repair mechanisms with cell hybrids can be uniquely carried out in our combined laboratories.

The complex experimental interactions necessary to a genetic analysis of human DNA repair require that a somatic cell genetics laboratory and a DNA repair laboratory work simultineously on the same passage of hybrid cells. This is due to the fact that proliferating human x mouse cell hybrids may continue to lose human chromosomes as they are passaged for extended periods or after they have been removed from storage in liquid nitrogen. If the genetic analysis is performed on one passage of a primary hybrid clone and the DNA repair assays are performed on another passage of that same clone, it will not be legitimate to compare the data since the human chromosome complement in the hybrid cell may have changed.

Dr. Lalley's group performs cytogenetic and enzymatic analysis in the hybrid cells while the same cells are undergoing analyses for DNA repair in Dr. Regan's laboratory. This allows for a rapid and accurate correlation of data which is absolutely necessary for successfully performing this work.

There are several excellent laboratories throughout the country devoted to DNA repair studies or to cell hybrid studies. However, we know of no other laboratory situation where genetic analysis of hybrids and DNA repair studies on the same passage of hybrids is being conducted in such a closely-knit fashion.

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BACKGROUND

A large amount of experimental evidence exists which indicates that damage to DNA is mutagenic and that physical and chemical mutagens can be carcinogens (14,15). It is evident, therefore, that the mechanisms by which cells repair damage to their DNA play a crucial role in carcinogenesis such that inefficient or inaccurate repair should correlate with increased rates of carcinogenesis. Support for this hypothesis is based on several observations: a) a correlation between the ability of physical and chemical agents to damage DNA and to induce cancer (16); b) the relationship between defective repair of 0^6 alkylated guanine and the induction of tumors by ethyl nitrosourea (17); and c) the photoreactivation of UV-induced tumors in fish (18). However, the most direct evidence that damage to DNA can be carcinogenic is derived from the existence of the recessively inherited human disorder, xeroderma pigmentosum (XP), in which there has been established a direct linkage between deficient ability to repair DNA damage and carcinogenesis (1-7). The outstanding clinical feature of XP is a very high prevalence of skin cancer, including basal and squamous cell carcinomas and malignant melanomas (6). Cleaver demonstrated that the biochemical lesion in XP cells was a defect in the excision pathway of repair of ultraviolet damage of DNA (2). Subsequent work has indicated that the defect in excision seems to be at the level of endonucleolytic incision of UV-irradiated DNA (7,19,20). In addition, XP cells in culture show increased mutation rates at lower doses of carcinogens in comparison to normal cells (5), thereby demonstrating a direct connection between DNA damage, mutagenesis and carcinogenesis.

While the molecular biology of this system has been advanced during the past ten years, the genetic basis is not well characterized. Studies utilizing the fusion of XP cells from different patients indicate that excision defective cells can be classified into six different complementation groups designated A through F (6). This genetic heterogeneity in XP is perplexing since the biochemical lesion is thought to be in the UV endonuclease which initiates excision of UV-induced pyrimidine dimers in DNA. Do these complementation groups indicate that: a) the enzyme is a polymeric protein with subunits coded for by different genes; b) they reflect genes coding for enzymes in a common pathway; c) they are the result of intragenic complementation; or d) the fusion of XP cells itself alters gene expression in an epigenetic way such that the number of complementation group does not indicate the number of gene loci involved? It should be noted here that extracts of XP cells although unable to excise dimers from their own chromatin can excise dimers from exogenous DNA. Normal cells are capable of both (21). In addition, studies done on mouse skin fibroblasts and epithelial cells and mouse embryo cells in culture indiate that the ability to perform excision repair of pyrimidine dimers

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is partially lost during development (8,22-24). These data suggest that the XP phenotype may be caused by mutations in control genes which suppress the repair pathways rather than in structural genes for proteins. It is imperative to distinguish among these possibilities if the functional relationships between DNA repair, mutagenesis and carcinogenesis are to be fully understood.

These questions can be investigated by employing the unique and powerful techniques of somatic cell hybridization. This strategy has made it possible to incorporate two different genomes in the same cell, map human genes (9) and mouse genes (25) to specific chromosomes, and dissect complex enzyme systems (11,12). In this parasexual technique, cells are fused with a membrane fusion agent such as inactivated Sendai virus or polyethylene glycol. Parental cells, deficient for specific enzymes, are selected against with selection media. Hybrid cells, whose nuclei fuse and complement the parental cells' deficiencies, survive. Employing standard tissue culture techniques, hybrid cells can be cloned, grown and harvested for analysis of specific biochemical markers. The cell hybridization procedures have been thoroughly described (9,13). The important characteristics of interspecific man x rodent somatic cell hybrids are: human but not mouse chromosomes are preferentially lost (26); individual chromosomes can be identified by appropriate banding and staining techniques (27) and because of evolutionary differences, a large number of homologous enzymes in man and mouse differ electrophoretically and thereby serve as gene markers in cell hybrids (13). Thus, proliferating cell hybrids with different numbers and combinations of human chromosome can be isolated providing a system of chromosome segregation necessary to map genes to specific chromosomes and to genetically dissect complex enzyme systems.

The partial genetic complements retained by independent hybrid clones can be correlated with the expression of any phenotype which is distinguishable between parental cells. If two or more phenotypes are expressed concordantly in a series of independent hybrid clones, the genes coding for these phenotypes are assumed to be on the same chromosome. Similarly, if a phenotype is concordantly expressed with a specific chromosome, then its gene can be assigned to that chromosome. Employing this strategy, over 75 enzyme loci have been mapped in man (10,13). Since the magnitude of DNA repair in the mouse is less than 10% of the excision repair seen in human cells, repair in man x rodent somatic cell hybrids can be readily characterized as human or mouse (8,22). Therefore, this parasexual technique of human gene mapping can be applied to mapping the genes required for DNA repair in man.

As described above, man x mouse somatic cell hybrids are uniquely suited for genetically dissecting complex enzyme systems by isolating their component parts. This allows for the investigation of the individual contribution made by structural genes, processing genes and

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control genes to the final phenotypic expression of an enzyme or enzyme system. Using this procedure, Lalley et al.(11, 12) demonstrated that β-hexosaminidase A (HEX_A), the enzyme deficient in Tay-Sachs disease, was composed of subunits coded for by different genes located on different chromosomes and that the expression of HEX_A was dependent on the expression of both genes. Processing genes, which function in the posttranslational modification of enzymes, have been demonstrated for acid phosphatase (28). Champion and Shows (29) demonstrated the existence of a gene which effects the final expression of several lysosomal enzymes and is defective in mucolipidosis II (I-cell disease). The control of gene expression in cell hybrids has been demonstrated by Peterson and Weiss (30) and Malawista and Weiss (31) who described the activation of mouse albumin in mouse x rat hepatoma cell hybrids.

The studies described above demonstrate the feasibility of utilizing the somatic cell hybrid approach to investigate the genetic structure of DNA repair mechanisms in man. A preliminary study was carried out to confirm this.

Twenty-four man x mouse hybrids segregating human chromosomes have been analyzed for the ability to repair DNA damage following UV irradiation. Our preliminary results demonstrate that it is possible to distinquish the human DNA repair components from mouse DNA repair components in the human x mouse somatic cell hybrids (see figure). The hybrids assayed for repair thus far appear to group themselves into one of three categories: (a) those having a magnitude of repair similar to human cells (b) those having mouse-like repair, and (c) hybrids intermediate between the two.

Segregation of the ability to repair UV damage in these 24 hybrids has been tested for linkage with 28 enzyme markers representing genes known to be assigned to 20 of the 24 different human chromosomes. Concordant segregation was not observed between DNA repair and these other enzyme markers. These preliminary data indicate that the assignment of a gene(s) required for the ability to repair UV-induced DNA damage can be excluded from several human chromosomes. Further studies, including cytogenetic analysis of selected clones, need to be conducted in order to positively identify the human chromosome(s) which encode(s) the gene or genes required for DNA repair in man. These data warrant an assiduous pursual of this project with the objective being as definitive a genetic analysis of human repair as the system will allow.

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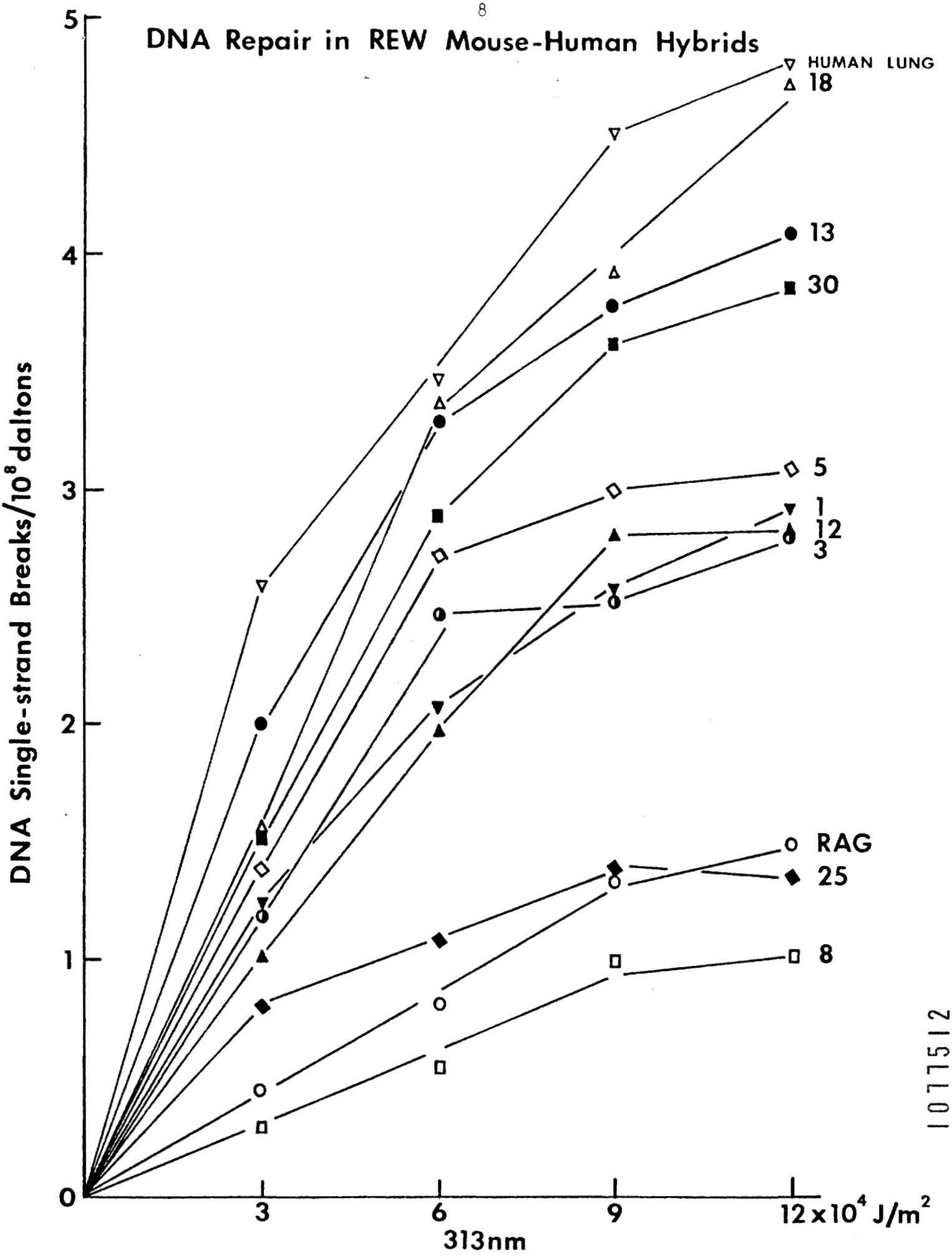
DNA Repair in REW Mouse-Human Hybrids
DNA Single-strand Breaks/10^8 daltons
0
1
2
3
4
5
3
6
9
12×10^4 J/m^2
313nm
HUMAN LUNG
18
13
30
5
1
12
3
RAG
25
8

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Figure 1 DNA repair in primary mouse x human somatic cell hybrid clones (REW) as measured by the dBrU photolysis assay. Hybrid clones were derived from a fusion of mouse RAG cells, derived from a renal adenocarcinoma of the BALB/c mouse, and human WI-38 embryonic lung fibroblasts. All cells received 20 J/m^2 of 254 nm UV radiation followed by a 20 hour repair period prior to the assay. Three distinct levels of repair were observed: human (13, 18 and 30), mouse (8, 25 and a Rag control) and an intermediate level of repair (1, 3, 5 and 12).

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EXPERIMENTAL METHODS

1. Cell Culture

a) Human Cell Lines

Human parental cells will consist of WI-38, a karyotypically normal lung fibroblast line; several x-autosome translocation lines useful in regional chromosome mapping studies; A549, a tumor cell line initiated from a human alveolar cell carcinoma; and XP fibroblasts from five complementation groups obtained from the American Type Culture Collection. All the cells needed are either presently growing or stored in our liquid nitrogen cell bank.

b) Mouse Cell Lines

Parental mouse cell lines to be used consist of the RAG line which is deficient for hypoxanthine phosphoribosyltransferase ($HPRT^-$) and the $LM/TK^-(TK^-)$ line which lacks thymidine kinase. These cells die in HAT medium. For certain hybridizations mouse bond marrow or spleen cells will be utilized. These cells will be taken from the animal and fused directly to the human cells without being put in culture.

All cells are grown under optimal conditions with respect to type of medium, percent of serum supplement and CO_2 tension. Cells are routinely checked for mycoplasma and other contaminations.

For enzyme analysis, cell homogenates are prepared from confluent monolayers. Cells are washed three times in cold serum free media and resuspended in 0.05M TRIS, pH 7.4 at a concentration of 70×10^6 cells/ml. Cells are disrupted either by freeze-thawing five times or mechanically in a Potter-Elvehjem tissue grinder, and centrifuged at 4° for one hour at 40,000 g.

For repair assays, the cells are counted using an electronic laser beam counter, planted in plastic petri dishes, incubated for 48 hours in growth medium, and labeled by incubating them for about 20 hours in medium containing radioactive DNA precursors. Cells for bromodeoxyuridine photolysis assays are labeled with ^{3}H-thymidine (1.9 Ci/mmole) at a concentration of 4 μCi/ml and ^{14}C-thymidine (500 mCi/mmole) at a concentration of 0.5 μCi/ml; cells for dimer analysis are labeled with ^{3}H-thymidine (50 Ci/mmole) at a concentration of 0.1 μCi/ml and ^{14}C-uridine (58 Ci/mmole) at a concentration of 1 μCi/ml. After the labeling period, cells are incubated in nonradioactive growth medium for 2-3 hours.

2. Cell Hybridization

Somatic cell hybridization will be accomplished by fusing parental cell lines either in suspension or in monolayers with β-propriolactone, inactivated Sendai virus or polyethylene glycol (PEG) as fusion agents (32,33). Hybrid cells are to be isolated using the HAT selection system of Littlefield (34). In HAT selection medium, deficient mouse cell lines fail to grow, human fibroblasts grow slowly while hybrid cells proliferate. Individual hybrid clones will be isolated as described (11). Hybrid clones are to be expanded, harvested and examined for specific biochemical markers, ability to repair DNA damage and retention of specific human chromosomes. All independent hybrid clones isolated under these conditions lose human chromosomes, but retain all the mouse chromosomes.

It is also possible to generate hybrids which segregate mouse chromosomes, but retain all the human chromosomes (35,36). This can be accomplished by utilizing differentiated mouse cells such as bone marrow cells or spleen cells as the mouse input cells, and established human cell lines as the human input cells. The fusion procedure is the same as described above. Following fusion, the cells are grown in selection medium. The mouse parental cells will not attach to the surface of the culture flask and grow, and be removed with subsequent changes of growth medium. The human parental cells will die in the selection system, or proliferate slowly. Hybrid cells can be isolated as described. Under these conditions, the hybrid clones preferentially segregate mouse chromosomes.

3. Detection of DNA Single-Strand Breaks on Alkaline Sucrose Gradients

Cells with radioactive labeled DNA are suspended in saline - 0.12% EDTA at a maximum concentration of 10^5 cells/ml. Ten thousand cells (50 μl) are lysed for 1 hour at room temperature in 200 μl of 1N NaOH overlayed on 5-20%, 4 ml alkaline sucrose gradients containing 2M NaCl. Gradients are centrifuged at 30,000 rpm for 150 minutes in a SW-56 rotor in a Beckman ultracentrifuge. Seven-drop fractions are pumped onto strips of filter paper using a Technicon proportioning pump. Strips are dried, given a 1 hour wash in 5% TCA and two 30 minute washes in 95% ethanol. Acid insoluble radioactivity is measured in a toluene-based scintillator in a Packard or Searle liquid scintillation counter. The distributions of radioactivity are converted to weight average molecular weights by a computer program based on the distances sedimented by phage DNAs of known molecular weights: T4 DNA, 55 X 10^6; λDNA, 15 X 10^6; and σX174 DNA, 1.7 X 10^6. The number of single-strand DNA breaks in any sample is calculated by comparing the reciprocal values of control (untreated) DNA and experimental (UV-irradiated, carcinogen exposed, etc.) DNA using the following formula:

$$\#\text{ of breaks } \left(2\Delta\frac{1}{M_w}\right) = 2[1/M_{w(\text{treated})} - 1/M_{w(\text{control})}].$$

Strand break numbers may be calculated from data on different single-label gradients (cells labeled with ^{3}H-thymidine) or from a double-label gradient containing treated (^{3}H-labeled) cells and an internal control (^{14}C-labeled) cell population.

4. The dBrU Photolysis Assay

The usual procedure for this assay utilizes cells which have been prelabeled with 0.5 μCi/ml [^{14}C]-thymidine (500 mCi/mmol) or 4 μCi/ml [Me-^{3}H]-thimidine (1.9 Ci/mmol). Prior to any insult, the media containing radioactive label was replaced with original growth media and incubated for 2 hours. Two dishes of cells were needed for each assay, one labeled with ^{14}C and one with ^{3}H as above. Immediately following treatment of both dishes with the insult (UV light, gamma rays, chemical agents, etc.), thymidine and dBrU (10^{-4}m) were added to the ^{14}C and ^{3}H labeled cells, respectively. These cells then incubated for the chosen repair period, usually 18-20 hours, thus allowing the incorporation of dBrU in the repair patch of the ^{3}H labeled DNA. The cells were then harvested and mixed at a concentration of 2 X 10^5 cells/ml. An aliquot of this cell mixture was placed in a quartz cuvette and given a series of 313 nm radiation doses from a Hilger quartz prism monochromator with a 1000 watt high pressure mercury arc source. This radiation sensitizes the dBrU containing patches to alkaline sucrose gradients. About 10^4 cells from each dose of 313 nm radiation were analyzed for single-strand breaks on alkaline sucrose gradients as previously described. The gradients were fractionated on filter paper strips which were then washed and counted for double-label in a liquid scintillation system. The resulting data were analyzed by a PDP-11 computer which plots the radioactivity profiles and calculates the molecular weights of the ^{14}C and ^{3}H labeled DNAs. The differences of the reciprocal molecular weights (Δ1/Mw) were plotted as a function of the 313 nm light fluence. The number of resulting breaks for any given fluence of 313 can be calculated as 2 X Δ 1/Mw for that fluence. The total number of repaired sites detected can be estimated as 2 X Δ 1/Mw at 313 nm light saturation.

Given the following:

N = total number of repaired regions/10^8 daltons
n = number of dBrU residues per region
F = 313 nm light fluence in ergs/mm^2
B = number of breaks or repaired regions for a given 313 nm fluence/10^8 daltons
α = 7.6 X 10^{-8} mm^2/erg - constant related to DNA cross section calculated from data on fully substituted DNA

The probability of 313 nm radiation making a break in a given repaired region is: $1 - e^{-n\alpha F}$. If n is large such as in long patch excision repair, then 313 nm light saturation will be reached; N can be estimated and the patch size estimated through the calculation of n in: $\frac{dB}{dF} = Nn\alpha$.

5. Direct Measurement of Thymine-containing Dimers in UV-irradiated Cellular DNA

An aliquot of the isolated DNA containing 100,000 to 500,000 cpm of ^{3}H-thymidine is precipitated with 5% TCA, washed with 95% ethanol, and dried. To hydrolysate the DNA to the free bases and dimers, the DNA is heated to 175° in sealed tubes containing 0.2 ml of 90% formic acid. The dimer analysis is described in detail by Carrier and Setlow (Meth. Enzy. 21: 230-237, 1971). The hydrolysate is spotted onto sheets of Whatman #1 paper, developed in two dimensions to separate the thymine-containing dimers from thymine. The thymine and dimer regions are cut out, placed into vials with a dioxane based counting solution, and the radioactivity determined. The percent dimers are calculated as $\frac{\text{thymine}}{\text{thymine-containing dimers}} \times 100$.

6. Enzyme Analysis

Primary hybrid clones will be analyzed for ability to repair UV-induced DNA damage, and the expression of 35 enzyme markers representing all of the human chromosomes except the Y. The chromosome assignment of the genes coding for these enzymes has been summarized (10,13,37). Enzyme and electrophoretic procedures will be carried out as previously described (11,13,38).

7. Karyotype Analysis

Karyotypic analysis of hybrid clones will performed on parallel cultures used for enzyme analysis and DNA repair assays. Giemsa - 9, Giemsa - trypsin and constitutive heterochromatin C-banding techniques will be employed to identify the chromosomes as described (27,40,41).

8. Prereplication Repair in Man x Mouse Somatic Cell Hybrids Following UV Radiation:

All human and mouse parental input cell lines will be tested for their ability to repair DNA following UV radiation. If any of the parental input lines exhibit abnormal repair profiles, hybrid lines derived from these cells will not be employed for primary gene assignment experiments. Previous work showed that mouse cells have only 10%

1077517

of the human excision capacity and that it is possible to distinguish the human DNA repair components in the human x mouse somatic cell hybrids (8). Therefore, man x mouse hybrid clones will be analyzed for DNA repair capacity following UV radiation and classified as positive or negative for human DNA repair capacity indicating that the hybrids have retained or lost the human genes coding for this function. The ability to repair UV damage will be compared to the presence or absence of 35 human enzyme markers. Genes coding for these enzyme markers have been assigned to each human chromosome except the Y chromosome. Concordant segregation of the ability to repair UV damage with specific gene markers will determine the gene-gene linkage relationship, and by implication the chromosome assignment(s) of the gene(s) involved in DNA repair in man. Karyotypic analysis of selected clones will be performed in order to confirm the assignments. The three goals of this experiment are: (a) to determine the amount of DNA repair in hybrid cells; (b) to determine the number of genes required to carry out this process; and (c) to assign these genes to specific human chromosomes. The determination of the amount of DNA repair relates to a gene dosage effect. Many of the hybrids will be functionally haploid for the human chromosomes which they retain since they will retain only one homologue of a homologous chromosome pair and thus have only one copy of the genes located on that chromosome. Other hybrids will retain both homologues and be functionally diploid and have two copies of the genes located on that chromosome pair, while still other hybrids will have lost both homologues and be deficient for the genes located on that chromosome pair. If the amount of DNA repair is gene dosage dependent, then three classes of hybrids should be found: (a) hybrids negative for human DNA repair; (b) hybrids possessing 50-60% of the normal human excision capacity; (c) hybrids possessing 100% of the normal human excision capacity. The negative hybrids would be analogous to XP patients, hybrids in the second class would be analogous to heterozygotes for DNA repair, and hybrids in the third class would be analogous to individuals normal for DNA repair. If DNA repair in not gene dosage dependent, then only negative hybrids and hybrids expressing 100% of normal human excision capacity should be found. A third explanation is that the hybrid clones are heterogenous for the chromosomes retained (25). Differentiation between these three possibilities will have important implications for determining individuals heterozygous for xeroderma pigmentosum and for prenatal diagnosis of this genetic defect. Determination of the number of genes required to carry out DNA repair of UV-induced damage and the chromosomal assignment of these genes will help to dissect the interreactions of the several enzymes involved in UV repair.

1077518

9. Formation of Man x Mouse Somatic Cell Hybrids Utilizing Different XP Cells as the Human Input Cells:

An important question that must be answered is whether or not the mouse input cells can complement defective DNA repair in human cells. Therefore, man x mouse somatic cell hybrids will be generated employing human cells derived from five different XP complementation groups. The biochemical defect in excision repair defective XP cells has been postulated as the UV endonuclease which initiates excision of UV-induced pyrimidine dimers in DNA. The existence of five complementation groups defining one enzymatic step has not been satisfactorily interpreted, but may suggest that there are multiple subunits for human UV endonuclease, or that the process of cell hybridization alters gene expression such that complementation *in vitro* is not a simple indicator of the number of different gene loci involved. There are several examples in the literature where enzyme defects in one species were complemented by the formation of a heteropolymer with polypeptide subunits supplied by another species (13, 41). On the other hand, there are several examples where cell fusion has altered gene expression (30,31). If human DNA repair is complemented in XP x mouse hybrids, then the nature of this complementation can be determined by generating XP x mouse hybrids which segregate mouse chromosomes. If the mouse is supplying a specific gene product, the loss of human DNA repair capacity should correlate with the loss of a specific mouse chromosome. If complementation is an epigenetic effect, then no such correlation will be seen. These studies will help to elucidate the precise genetic defect in xeroderma pigmentosum.

1077519

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1077520

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1077521

TECHNICAL PROPOSAL

DIRECT COSTS

	Year 01		Year 02		Year 03	
	PY	$	PY	$	PY	$
Personnel Salaries and Wages	1.6	33,800	1.6	36,200	1.6	38,700
Travel		500		500		500
Materials and Supplies		11,000		12,000		13,000
Crafts Maintenance Service		500		600		700
Glassware Washing		1,000		1,200		1,400
Technical Information		500		500		500
Total Direct Costs	1.6	47,300	1.6	51,000	1.6	54,800

BUDGET COMMENTS

Personnel:

Peter A. Lalley	Ph. D.	Senior Investigator
James D. Regan	Ph. D.	Senior Investigator
Technician	to be named	

Fringe Benefits:

26% of salaries

Travel:

Funds are requested to support participation in related scientific meetings, trips to Bethesda to confer and participate in workshops, etc.

Materials and Supplies:

Includes cost of non-capital equipment, tissue culture (media, sera, antibiotics, plasticware, filters, special glassware, liquid nitrogen, etc.) and substores supplies (glassware, general chemicals, alcohol, surgical gloves, instruments, paper towels, etc.), chemicals for enzyme strains, and labeled substrates.

Craft Maintenance Service

For repair and service of existing laboratory facilities and instruments.

1077522

TECHNICAL PROPOSAL

Glassware Washing

Includes the cost of labor and materials involved in cleaning and sterilizing of laboratory glassware.

Technical Information

Cost of editing, composing, and reproducing business-related items such as proposals, reports, etc. are costed on a usage basis.

1077523

BUSINESS
MANAGEMENT COST PROPOSAL
For
UNSOLICITED PROPOSAL

Date of Submission:	December 6, 1978
Title of Proposed Project:	Genetic Analysis of DNA Repair in Man with Cell Hybrids
Co-Principle Investigators:	Dr. Peter A. Lalley Dr. James D. Regan
Institution:	Biology Division P. O. Box Y Oak Ridge National Laboratory Oak Ridge, Tennessee 37830
Proposed Level of Effort:	Peter A. Lalley: 20% of time James D. Regan : 10% of time
Total House of Other Support Personnel:	52 hrs/week

This proposal ( ) does, (X) does not involve recombinant DNA research

1077524

BUDGET COMMENTS

Utilities

Cost of electricity, steam and water utility operators to maintain the utility system. This project occupies approximately 700 ft.^{2} of laboratory and office space.

The work proposed in this project does not overlap or duplicate any other projects submitted in behalf of the personnel included in this proposal. Dr. Lalley's other project involves the use of somatic cell hybrids in a genetic analysis of the aryl hydrocarbon hydroxylase system and C-type RNA tumor virus infectivity and replication. Dr. Regan's other projects involve a molecular characterization of DNA repair systems, none of which utilize somatic cell hybrids.

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