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PRINCIPAL INVESTIGATOR: Wen-Hwa Lee, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Health Science Center
San Antonio, Texas 78284-7758

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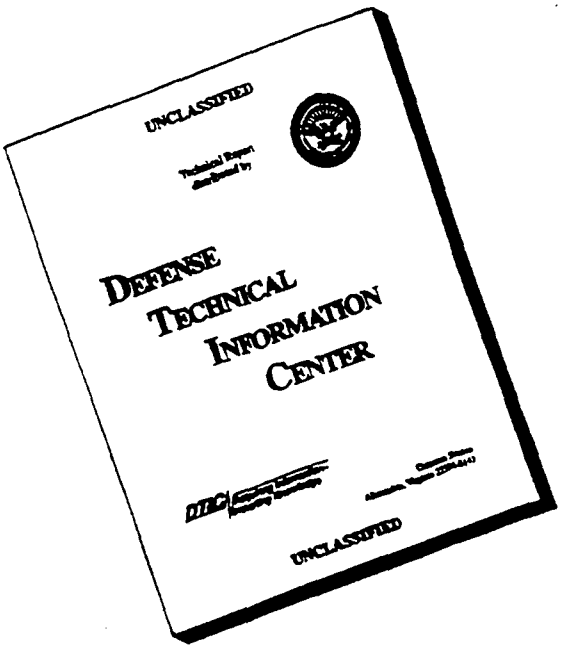

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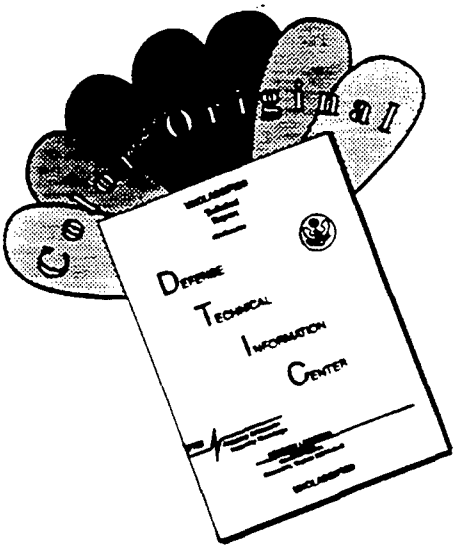

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13. ABSTRACT *(Maximum 200*

The objective of the program is to establish at the University of Texas Health Science Center at San Antonio an in-depth training program in the Molecular Genetics of Breast Cancer. The goal of the program is to train highly qualified pre-doctoral students in the genetic, cellular, and molecular basis of Breast Cancer. The training program is conducted within the Molecular Medicine Ph.D. Program by a select group of faculty whose research projects are relevant to breast cancer. Breast cancer meetings and Molecular Medicine Symposia are an integral part of the training program for students supported by the Breast Cancer Training Program. A major strength of the program is the high quality of the Program faculty, and the interactive nature of the Breast Cancer research community in San Antonio. The program faculty are organized into four subprograms, which encompass scientists studying different aspects of breast cancer and cancer therapy, as well as fundamental mechanisms of cell growth, differentiation and molecular genetics. During the reporting period, research by the six students supported by the training program resulted in the publication of five peer-reviewed articles. Thus the program is making excellent progress in the training of upper level students. Additionally the program continues to attract qualified students for entry into the program.

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PI - Signature Date

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INTRODUCTION

1. Brief Description of the Training Program and Its Objectives

The continued objective of the program is to establish at the University of Texas Health Science Center at San Antonio an in-depth training program in the Molecular Genetics of Breast Cancer. The most important goal of the program is to train highly qualified pre-doctoral students in the genetic, cellular, and molecular basis of Breast Cancer. It is our hope that with the background in Breast Cancer Biology that these students have obtained, they will provide the momentum and scientific expertise for significant discoveries in this field in the future. The training program is conducted within the Molecular Medicine Ph.D. Program by a select group of faculty whose research projects are relevant to breast cancer. An additional goal of the program is to promote synergistic interactions between the various laboratories engaged in breast cancer research. A major event to promote these interactions was the Port Aransas Research Conference where students [including 18 in-coming students] and breast cancer researchers plus others involved in basic research gathered to discuss their research programs. The agenda for this meeting is included in this report. Another important event is the Annual Breast Cancer Symposium held in San Antonio. All students supported by the program were required to attend. Finally, an outstanding Molecular Medicine Minisymposium Series was sponsored by the University of Texas Institute of Biotechnology. Three of these series, "Molecular Genetics of Cancer", "Signal Transduction and Cell Cycle" and "DNA Replication, DNA Repair and Genetic Recombination" dealt directly with issues related to cancer. The programs for these minisymposia are attached.

One of the major strengths of the program is the high quality of the Program faculty, and the interactive nature of the Breast Cancer research community in San Antonio. The program faculty are organized into four subprograms, which encompass scientists and physicians studying different aspects of breast cancer and cancer therapy, as well as fundamental mechanisms of cell growth, differentiation and molecular genetics. These faculty groupings are listed here, detailed descriptions of individual research programs were included in the original application.

A Breast Cancer Sub-Program

C. Kent Osborne, M.D.
John Chirgwin, Ph.D.
Suzanne Fuqua, Ph.D.
E. Lee, Ph.D.
W.-H. Lee, Ph.D.
Z. Dave Sharp

B. Growth Factor Sub-Program

Douglas Yee, M.D.
Gregory Mundy, M.D.
Robert J. Klebe, Ph.D.
Betty Sue Masters, Ph.D.

C. Drug Development Sub-Program

Daniel Von Hoff, M.D.

D. Molecular Genetics Sub-Program

Robin Leach, Ph.D.
Peter O'Connell, Ph.D.
Alan E. Tomkinson, Ph.D.
*Robert J. Christy, Ph.D.

* New Member of the Program Faculty

Each of these faculty members maintains an active research program. A listing of their research support is found below.

In this progress report, the relationship between the Breast Cancer Training Program and the Molecular Medicine Graduate Ph.D. Program is reviewed, and additional or updated information is provided regarding:

Research Support for Program Faculty
Listing of Supported Trainees
Project Summaries of upper level trainees
Changes to the Program Faculty:
Additions:
Robert J. Christy, Ph.D.
Biographical Sketch, Research Support, Project Summary

Removals:
Barbara Bowman, Ph.D.
Edward Seto, Ph.D.
Course Changes:
Appendix: Reprints of Trainee Publications

2. Relationship between the Breast Cancer Training Program and the Molecular Medicine Graduate Ph.D. Program

The Breast Cancer Training Program was implemented within the context of the Molecular Medicine Graduate Ph.D. Program. The Molecular Medicine Ph.D. Program is a recently established interdisciplinary Ph.D. training program in the Graduate School of Biomedical Sciences at the UTHSCSA. For the academic year 1995-96, there was a total of 26 students enrolled in the Molecular Medicine Ph.D. Program -- 24 Ph.D. and 2 M.S. Of those 25 students, only six are supported by the Training Program in the Molecular Basis of Breast Cancer.

The Breast Cancer Training program takes advantage of the internationally recognized breast cancer research program existent in the institution for many years, and offers a unique opportunity for students interested in starting careers in breast cancer research. The participating scientists in this breast cancer program represent diverse departments including the Divisions of Medical Oncology, Hematology and Endocrinology in the Department of Medicine, and the Departments of Cellular and Structural Biology, Pathology and Biochemistry. In addition, the new University of Texas Institute of Biotechnology and the San Antonio Cancer Institute [SACI], an NIH-designated Cancer Center, represent outstanding resources for training opportunities in clinical and basic science research. The national and international reputation of the participating faculty serve to attract a large

number of excellent applicants to the breast cancer research track in the Molecular Medicine program. The continuation of a Breast Cancer Specialized Program of Research Excellent (SPORE) grant to the institution documents the quality of breast cancer research available to trainees.

The rationale for administering the breast cancer training program in the Molecular Medicine Ph.D. program is based on several important criteria: [1] The Molecular Medicine curriculum is specifically designed to provide basic science training while integrating fundamental principles of molecular biology with modern medicine. A Molecular Medicine Core course provides students with the mechanisms underlying human disease and provides intensive review of specific diseases [including breast cancer] that may serve as models for how human diseases can be studied at the molecular genetic level. [2] The Molecular Medicine program requires the participation of both clinical and basic scientists in the training process. The inclusion of MDs on all student advisory committees insures that every graduate has a clear perspective on the clinical relevance of the basic research in their program, that in most instances, will serve as a guide for the project. [3] The Molecular Medicine program is an interdepartmental, interdisciplinary program that offers flexibility to students in terms of research laboratories, advisors and committee members. This arrangement offers a real potential for synergism in breast cancer research not possible in traditional department-bound programs. In summary, our program offers a near perfect environment for Ph.D. training in breast cancer and has attracted many well-qualified applicants.

3. Research Support for Program Faculty

An essential component of maintaining a successful and aggressive training program in Breast Cancer Research is the continued research funding of the individual Program Faculty laboratories. Current funding for each member of the Program faculty is detailed in Table 1. As can be readily seen from the table, the faculty have been extremely successful in obtaining research funding, including over $14,880,628 in direct costs for the 1995-1996 fiscal year. ***(See Research Support Table, Page 14)***

4. Listing of Supported Trainees

Trainees receiving support from the Training Program in the Molecular Basis of Breast Cancer Research are selected from among entering first year students in the Molecular Medicine Ph.D. Graduate Program. In subsequent years of their training, they may be maintained on the Training Program, or transferred to other funding sources, depending on the nature of their research interests, and the availability of grant support. The following trainees were supported on the Breast Cancer Training Program

1995-1996

Upper Level Students

Linda DeGraffenried
Jennifer Gooch
David Levin
*Shang Li
Zachary Mackey
Ernesto Salcedo

* New to the program this year, see report below.

Record of Previous Year's Trainees:

Jim Fitzgerald	Graduated from the program with an M.S. degree.
Christa Hargraves	Left the program for academic reasons.
Zachary Mackey	Continues in the program as an upper level student [see report below].
Harold Pestana	Left the program for academic reasons.
Yuewei Qian	Graduated from the from the program with a Ph.D. Postdoc in James Maller's laboratory at the Howard Hughes Medical Institute at The University of Colorado School of Medicine. Dr. Qian's research involves understanding the cell cycle and cell proliferation. This is a problem that is relevant to all types of cancer, including those of the breast.
James Wang	Continues in the Molecular Medicine Ph.D. program, currently funded by advisor's grant. Although no longer in the Training program, his work on the mechanism of viral latency is important in some cancers. James presented his work at the Port Aransas Research Conference on Breast Cancer.
Linda deGraffenried	Continues in the program as an upper level student [see report below].
Jennifer Gooch	Continues in the program as an upper level student [see report below].
David Levin	Continues in the program as an upper level student [see report below].
Ernesto Salcedo	Continues in the program as an upper level student [see report below]. As of this report date [10/22/96] however, Ernestor was removed from the training grant since he elected to pursue work in a non-program facutly's laboratory [Dr. Steve Britt]. He was replaced with another highly qualfied student to be reported in the next cycle.

The 1995-1996 academic year marks the third full year of operation for the Molecular Medicine Ph.D. Program, and the second for the Training Program in the Molecular Basis of Breast Cancer Research. The availability of highly qualified applicants to the Molecular Medicine Program has proven to be excellent. Over 150 applications were received for admission to the Fall 1995 entering class. Eight students began classes in August of 1995. The total number of students at the start of the Fall semester, 1995 the Molecular Medicine Ph.D. Program at all levels was 26, which includes 12 women, and 3 minorities (1 black, 2 Hispanic students). All three minority students were supported by the Training Program in the Molecular Basis of Breast Cancer Research.

5. Project Summaries of Upper Level Trainees

Linda DeGraffenried ***Mentor -- Dr. Suzanne Fuqua***

Tamoxifen is an effective therapy for estrogen receptor (ER)-positive breast cancer patients, however almost all women will eventually become resistant and fail this hormonal therapy. Clinical data suggests that in some patients, tamoxifen might actually stimulate tumor proliferation. To understand one potential mechanism for the stimulatory effects of tamoxifen, we have studied regulation of the rat prolactin promoter because tamoxifen is known to increase

prolactin levels in rat pituitary cell lines. The rat prolactin promoter contains four pit-1 transcription factor binding sites which are important in its regulation. In addition, there is a nonconsensus estrogen response element in the proximal region of the promoter which may play a role in the hormonal regulation of this gene. We have analyzed the hormonal regulation of the rat prolactin promoter using transient transactivation assays in human breast cancer cells. In the absence of pit-1 expression, the rat prolactin promoter was induced by tamoxifen ten-fold in ER-positive MCF-7 cells, but not ER-negative MDA-MB-231 cells. Estrogen did not induce this promoter in the absence of pit-1. In the presence of pit-1, the rat prolactin promoter was induced by estrogen, but not tamoxifen in MCF-7 cells. We are currently examining whether tamoxifen stimulation of the rat prolactin is working through a classical estrogen response pathway, or a novel mechanism such as a putative AP-1 site buried within the four pit-1 binding sites. We hypothesize that the rat prolactin promoter may serve as a model for cell and type-specific tamoxifen agonist effects in human breast cancer cells.

This project is directly relevant to breast cancer. Elucidating the basis for tamoxifen resistant could lead to ways to augment the use of this important therapeutic agent and save the lives of thousands of afflicted women.

Jennifer Gooch ***Mentor -- Dr. Douglas Yee***

Dr. Yee's laboratory is interested in the growth regulation of breast cancer cells by insulin-like growth factors (IGFs). Data from several laboratories had suggested that interleukin-4 (IL-4) and IGFs share common signaling pathways. Since it was known that IL-4 could directly inhibit breast cancer cell proliferation, Jennifer began examining the potential overlap of growth stimulatory and growth inhibitory signaling pathways in breast cancer cells.

She first confirmed that IL-4 was inhibitory for breast cancer cells. Furthermore, she showed that IL-4 treatment of several breast cancer cell lines resulted in apoptosis as measured by the TUNEL assay. Using Northern analysis, she was able to show that expression of IL-4 receptor was ubiquitous in human breast cancer cells. Treatment of cells with IL-4, IGF-I, or insulin all resulted in phosphorylation of insulin-receptor substrate-1 (IRS-1), however, generation of signals distal to this (MAP kinase) were not clearly propagated by IL-4. Thus, differences in IL-4's growth inhibitory effects and IGF-I's growth stimulatory effects may be due to differences in the activation of signaling pathways distal to IRS-1. She is currently studying the dose response of breast cancer cells to IL-4, is beginning neutralization experiments, and has created a FLAG epitope tagged IL-4 receptor to transfect into breast cancer cells. The goal of her current work is to characterize the IL-4 signaling pathway in breast cancer cells that leads to apoptosis. As a corollary to this plan, she hopes to overexpress the IL-4 receptor in these cells to sensitize them to programmed cell death.

This project is relevant to breast cancer since intracellular signalling pathways are almost certainly involved in the growth stimulation at some stage of mammary cell tumor development or progression. The IGF-I and IL-4-IRS-1 pathway is an excellent candidate for such a pathway.

David Levin ***Mentor -- Dr. Alan Tomkinson***

DNA joining events are required to maintain the integrity of the genome. Three human genes encoding DNA ligases have been identified. In this project we are intending to identify the cellular functions involving the product of the LIG1 gene. Previous studies have implicated DNA ligase I in DNA replication and some pathways of DNA repair. During DNA replication, DNA ligase I presumably functions to join Okazaki fragments. However, under physiological salt conditions, DNA ligase I does not interact with DNA. It is our working hypothesis that DNA ligase I involvement in different DNA metabolic pathways is mediated by specific protein-protein

interactions which serve to recruit DNA ligase I to the DNA substrate. To detect proteins that bind to DNA ligase I we have fractionated a HeLa nuclear extract by DNA ligase I affinity chromatography. PCNA was specifically retained by the DNA ligase I matrix. To confirm that DNA ligase I and PCNA interact directly, we have found that in vitro translated and purified recombinant PCNA bind to the DNA ligase I matrix. In similar experiments, we have shown that DNA ligase I interacts with a GST (glutathione S transferase)-PCNA fusion protein but not with GST. Using in vitro translated deleted versions of DNA ligase I, we have determined that the amino terminal 120 residues of this polypeptide are required for the interaction with PCNA. A manuscript describing these studies is being prepared for publication.

This project is relevant to breast cancer since problems with DNA replication and repair will undoubtedly be involved in the development of all tumors at some stage in their progression.

Publications:

Mackey, Z.B., Ramos, W., **Levin, D.S.**, Walter, C.A., McCarrey, J.R. and Tomkinson, A.E. An alternative splicing event, which occurs in mouse pachytene spermatocytes, generates a form of DNA ligase III with distinct biochemical properties that may function in meiotic recombination. *Molec. Cell. Biol.* Accepted subject to revision.

Shang Li ***Mentor -- Dr. Wen-Hwa Lee***

Mutations of the *BRCA1* gene predisposes women to the development of breast cancer. The *BRCA1* gene product [BRCA1] is a nuclear phosphoprotein that is mislocated to the cytoplasm of breast cancer cells. To understand the basis of its cellular partitioning and function, Shang's project is to identify BRCA1-interacting proteins, confirm their *in vivo* interactions and to elucidate their relevance to the development of breast cancer. First, site-directed mutagenesis identified the functional nuclear localization sequence of BRCA1. Second, Shang used the yeast two-hybrid assay to identify BRCA1-interacting proteins. From a human B-lymphocyte cDNA library, Shang identified four clones [hBRAPs] that encode polypeptides capable of interacting with BRCA1. When compared to the currently available GenBank, he found that one is novel, one has homology to an uncharacterized zinc-finger domain-containing protein, and two bear sequence homology to previously cloned cDNAs. Interestingly, the sequence of hBRAP21 is identical to that of the nuclear localization signal receptor hSRP1α, also known as importin-α or karyopherin-α. Shang is currently conducting in-depth analysis of the remaining clones, some of which appear to be nuclear transcription factors.

This project is directly relevant to breast cancer since it involves the study of a protein whose malfunction or mislocation leads to tumor development in the mammary gland.

Publications:

1. **Chen, C. -F., S. Li, Y. Chen**, P. -L. Chen, Z. D. Sharp, and W. -H. Lee. 1996. The nuclear localization sequences of the BRCA1 protein interact with the importin-α subunit of the nuclear transport signal receptor. J. Biol. Chem. In Press. *Note: The three authors in bold contributed equally to this work.*

2. Liu, C. Y., A. Flesken-Nikitin, **S. Li,** Y. Y. Zeng, and W. H. Lee. 1996. Inactivation of the mouse *Brca1* gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development. Genes Dev. **10**:1835-1843.

Ernesto Salcedo ***Mentor -- Dr. Barbara Bowman***

This project in Dr. Bowman's laboratory was a part of the investigation of Alzheimer's disease using a transgenic mouse model. Alzheimer's is a progressive neurological disorder primarily affecting geriatric populations. The progression of this disease is irreversible. The approach was genetic using the amyloid precursor protein [APP] on chromosome 21. Mutations in this gene are associated with early onset of the disease. Apoliprotein E [ApoE] is another gene implicated in Alzheimer's. Specifically, the ApoE4 isoform is correlated with late onset of the disease. The project was to use a transgenic mice model to test the hypothesis that the interaction between ApoE4 and APP670/671 mutant will produce the Alzheimer's disease [AD] phenotype. The transgenic mice used in the project expressed human ApoE and human APP in a murine knock-out background. My specific project was to conduct a behavioral assay known as the Morris Swim Test which is a test of memory. Although some positive results were obtained [i.e. behavior indicating memory impairment in test compared to control mice], the result were inconclusive due to small number [8] mice tested.

Zachary Mackey ***Mentor -- Alan Tomkinson***

DNA joining events are required to maintain the integrity of the genome. Three human genes encoding DNA ligases have been identified. In this project we are intending to identify the cellular functions involving the product of the LIG3 gene. Mammalian cell lines with reduced DNA ligase III activity exhibit spontaneous genetic instability and increased sensitivity to DNA damaging agents. We have cloned human and mouse cDNAs encoding DNA ligase III. In both mouse and humans, we have identified two forms of DNA ligase III cDNA that differ at their 3' ends and encode polypeptides with different C-termini. At the site where the cDNA sequences diverge, the nucleotide sequence resembles consensus splice donor/acceptor sequences. We have confirmed that these cDNAs represent alternatively spliced products from the same gene by cloning and analysis of the 3' end of the mouse LIG3 gene.

Analysis of DNA ligase III expression by northern blotting demonstrated that this gene is highly expressed in the testes. Using RT-PCR, we have examined the expression of the two forms of DNA ligase III cDNA in mouse tissues and cells. One form of DNA ligase III mRNA, DNA ligase III-a, is ubiquitously expressed. In contrast, expression of DNA ligase III-b mRNA is restricted to the testis. During spermatogenesis, DNA ligase III-b mRNA expression occurs during the latter stages of meiotic prophase. This restricted expression pattern suggests that DNA ligase III-b mRNA may have a specific role in the completion of meiotic recombination. In support of this idea we have shown that DNA ligase III-a interacts with the DNA strand break repair protein Xrcc1 whereas DNA ligase III-b does not. We suggest that the DNA ligase III-a/Xrcc1 complex functions in DNA repair in both somatic and germ cells whereas DNA ligase III-b functions in meiotic recombination. A manuscript describing these studies has been submitted to *Molecular and Cellular Biology*.

This project is relevant to breast cancer since genomic instability is likely to be involved at any of the several stages of breast cancer progression leading to malignancy. Methods to intervene and stabilize the genome could prevent progression and spread of the disease.

Publications:

Wang, Y.-C.J., Burkhart, W.A., **Mackey, Z.B.**, Moyer, M.B., Ramos, W., Husain, I., Chen, J., Besterman, J.M. and Tomkinson, A.E. Mammalian DNA ligase II is highly homologous with Vaccinia DNA ligase. *Journal of Biological Chemistry* 269, 31923-31928.(1994).

Husain, I., Tomkinson, A.E., Burkhart, W.A., Moyer, M .B., Ramos, W., **Mackey, Z.B.**, Besterman, J.M. and Chen, J. Purification and characterization of DNA ligase III from bovine testes. *Journal of Biological Chemistry* 270, 9683-9690 (1995).

Chen, J., Tomkinson, A.E., Ramos, W., **Mackey, Z.B.**, Danehower, S., Schultz, R.A., Besterman, J.M. and Husain, I. Mammalian DNA ligase III: Molecular cloning, chromosomal localization and involvement in meiotic recombination during spermatogenesis. *Molec. Cell. Biol.* 15, 5412-5422 (1995).

Mackey, Z.B., Ramos, W., Levin, D.S., Walter, C.A., McCarrey, J.R. and Tomkinson, A.E. An altrenative splicing event, which occurs in mouse pachytene spermatocytes, generates a form of DNA ligase III with distinct biochemical properties that may function in meiotic recombination. *Molec. Cell. Biol.* Accepted subject to revision.

6. Changes to the Program Faculty:

Additions: Robert J. Christy, Ph.D. to the Molecular Genetics Subprogram

Project Summary, Biographical Sketch, Research Support

Robert J. Christy, M.D.
Assistant Professor
Ph.D., The Johns Hopkins University

The primary research interest in my laboratory is the investigation of the genetic and metabolic regulation of gene expression. These studies utilize both *in vitro* and *in vivo* models to analyze gene expression in both adipose and liver. Using an in vitro culture system preadipocyte cells will differentiate into adipocytes in response to a specific hormonal stimuli. This terminal differentiation reflects the process of adipose tissue formation in vivo [including breast] and is mediated through increased rates of transcription of adipose-specific genes including fatty acid synthetase. We have found common DNA sequences in these lipogenic promoters that are important for activation during the determination and differentiation processes. The identification of the of the protein/DNA interactions involved in mediating this adipose-specific response is also underway. Other studies in the laboratory are investigating the effects diets and hormones on the regulation of lipogenic gene expression in liver and adipose tissue.

These studies are applicable to breast cancer since a major tissue-type in the breast is adipose. In addition, fatty acid synthetase is a prognostic molecule in tumors from breast cancer patients with markedly worse prognosis.

Removals from the faculty:

The faculty were saddened to loose one of our most distinguished members, Dr. Barbara Bowman, who passed away during this year. After many years of courageous battles, Dr. Bowman succumbed to breast cancer.

Dr. Edward Seto left the institution to assume a position at another university.

7. Course Changes: None this year.

CONCLUSIONS: The Breast Cancer Training Program has made excellent progress toward attracting and retaining excellently qualified students in breast cancer research. The students are receiving a high level of training in the modern research methods and theory. Combined with the basic instruction they receive in the Molecular Medicine Ph.D. Program, they will graduate as highly skilled researchers who will be very competitive for post doctoral positions in the premiere breast cancer laboratories in the world.

OTHER SUPPORT

PARTICIPATING FACULTY MEMBER	FUNDING AGENCY	IDENTIFYING NUMBER AND TITLE	PROJECT PERIOD	CURRENT YEAR DIRECT COSTS
Lee, W.-H.				
Active Support	Council for Tobacco Research	2992 Characterization of a Novel Cell Death Protein Regulated by Retinoblastoma Protein	07/01/96-06/30/97	78,261
	NIH/NEI	5R01EY05758-12 Molecular Mechanisms of the Retinoblastoma Gene	03/01/93-02/28/98	227,198
	NIH/NCI	2R01CA58318-03A1 Cancer Suppression by the Retinoblastoma Gene	05/01/95-04/30/98	165,552
	NIH/NCI	Translational Research in Breast Cancer - Developmental Project Do BRCA1-Deficient Mice Develop Breast Tumors? 2P50CA58183-05 C. Kent Osborne, P.I.	08/01/96-07/31/97	140,000
	NCI/NIH	1R01CA58183-04 SPORE in Breast Cancer, Project 5 Tumor Suppressor Genes in Breast Cancer Development C. Kent Osborne, P.I.	08/01/96-07/31/97	20,500
Osborne, C.K.				
Active Support	NIH	1PO1CA58183-04 Translational Research in Breast Cancer--San Antonio	09/30/92-09/29/95	1,499,312

	NIH	5P30 CA54174-04 San Antonio Cancer Institute- Program Leaders	08/01/94-07/31/98	1,411,266
	NIH	P01CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 3; Markers of Evolutionary Stages in Breast Cancer	07/28/92-02/28/97	76,246
	NIH	P01CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 5; TGF- alpha in the Pathogenesis of Breast Cancer	07/28/92-02/28/97	106,180
	NIH	P01CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 6; The IGF-System as a Potential Treatment Target in Breast Cancer	07/28/92-02/28/97	131,011
	NIH	1K12CA01723-03 Physician Scientist Training Grant in Oncology/Hematology	09/05/92-08/31/97	186,545
	NIH/NCI	5T32 CA0434-09 Training Program in Academic Medical Oncology/Hematology	05/01/93-04/30/98	64,300
	Susan G. Komen	Mechanisms of Tamoxifen Resistance	01/01/94-12/31/96	35,000
Pending Support	NIH/NCI	5P01CA30195-17 Markers of Breast Cancer Evolution and Progression, Program Project	03/01/97-02/28/02	1,814,795
	NIH	Target-Specific Contrast Agents for MR of Breast Cancer	12/01/96-11/30/99	228,756

Chirgwin, J.M.				
Active Support	VAMC	Merit Award Molecular Basis of Lysomal Targeting	03/01/93-02/29/97	78,506
	VAMC	Associate Research Career Scientist	04/01/94-03/31/98	41,351
Pending Support	VAMC	Merit Award Molecular Basis of Lysosomal Targeting	03/01/97-02/29/001	483,500
	NIH	R01	04/01/97-03/31/02	848,565
	NIH	R01	07/01/96-06/30/01	
	U.S. Army	Coagulation Factor XIIA Chain: Mechanism of Secretion	07/01/96-06/30/01	664,591
Christy, R.				
Active Support	NIH	5R29DK46360-04 Insulin Adipose Cell Commitment	05/01/93-04/30/98	70,000
	JDFI	JDFI 196014 Dietary and Hormonal Regulation of the Stearoyl-COA Desaturase 1 (SCDI) Gene	09/01/96-08/31/97	45,454
Fuqua, S.A.W.				
Active Support	NIH/NCI	5P50CA58183-04 SPORE in Breast Cancer, Project 2 Heat Shock Proteins and Drug Resistance	09/30/95-07/31/00	174,867
	NIH/NCI	PO1CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 2; Molecular Variants of Estrogen and Progesterone Receptor in Clinical Breast Cancer	07/28/92-02/28/97	116,533
	DAMD USARMC	New Mechanisms of Tamoxifen Resistance in Breast Cancer Patients	10/15/94-09/14/97	83,561
	DAMD	DAMD17-94-J-4112	01/01/95-10/14/97	83,166

	USARMC	Fellowship to Study the Involvement of Heat Shock Proteins in Drug Resistance in Human Breast Cancer		
	NIH	T32 CA70091-01 Training Program for Translational Breast Cancer	09/01/96-06/30/01	82,008
Pending Support	NIH/NCI	5 P01CA30195-17 Molecular Variants and Overxpression of ER in Clinical Breast Cancer Development	03/01/97-02/28/02	190,139
Klebe, R.J.				
Active Support	NIH	R01DE08144-07 Initial Events in Bone and Tooth Morphogenesis	07/01/88-06/30/97	133,054
Pending Support	USAMRMC	Unscheduled Expression of Metalloproteinases in Breast Cancer	10/01/97-02/28/00	299,941
Leach, R.J.				
Active Support	NIH/NCHGR	1P01HG00470-05 Saturation Mapping of Human Chromosome 3; Program Project Grant; Project 2	06/10/92-05/31/97	158,000
	NIH/NCHGR	R13HG00890-03 Third International Workshop on Chromosome 8	06/01/96-05/31/97	20,000
	NIH/NCHGR	R29AR40689-06 Regulation of Osteoblast-Specific Gene Expression	07/01/91-06/30/97	56,607
	NIH/NIDR	R03DE11848-01 Isolation of Candidate Genes for Amelogenesis Imperfecta	06/01/96-05/31/98	24,509

	Genetech	Relationship Between Myelination and Growth Hormone in a Mouse Hybrid	09/01/96-08/31/97	40,000
Pending Support	NIH/NIAMS	Pathobiology of the Osteoclast in Paget's Disease	04/01/97-03/31/02	149,359
	Morrison Trust	Identification of a New Gene Involved ir the Production of the Blood Chemical, Growth Hormone	10/01/96-09/30/97	33,292
	NIH/NICHD	Advances Towards a Mouse Model for the 18q-Syndrome	07/01/97-06/30/99	50,000
	ACS	Isolation of a Predisposition Gene for Paget Disease and Osteosarcoma	07/01/97-06/30/00	79,355
Lee, E. Y.-H.P.	NIH/NCI	1R29CA49649-09	07/01/94-04/30/99	145,455
Active Support		Tumor Suppression Function of RB and p53 in the Mammary Gland		
	NIH	HD30265-03	12/01/93-11/30/97	163,305
		Mouse Models for Studies of the Retinoblastoma Gene		
Masters, B.S.S.				
Active Support	NIH	R37HL30050	04/01/88-03/31/97	156,314
		Microsomal Electron Transport in Liver and Heart		
	Robert A. Welch Foundation	AQ1192 Structure-Function Relationships in the FAD- and FMN-Containing Enzymes, NADPH-Cytochrome P450 Reductase and Nitric Oxide Synthase	06/01/96-05/31/99	52,000
	NIH	R01GM31296-12	06/01/94-05/31/97	161,327
		Prostaglandin 19- and 20-Hydroxylation by Cytochrome P450		

	NIH	GM52419 Structural/Functional Modularity in Nitric Oxide Synthase	04/01/96-03/31/00	130,000
Mundy, G.R.				
Active Support	NIH	RR01346 General Clinical Research Center	12/01/93-11/30/98	223,187
	NIH	PO1CA40035 Effects of Tumors on the Skeleton	06/01/95-05/31/99	129,342
	NIH	P01CA40035 Effects of Tumors on the Skeleton Administrative Core	06/01/95-05/31/99	52,536
	NIH	R37AR28149 The Monocyte-Macrophage System and Bone Resorption	07/01/96-06/30/97	190,170
	NIH	PO1AR39529 The Osteoclast and its Regulation-Administrative Core	04/01/93-03/31/97	45,630
	NIH	PO1AR39529 The Osteoclast and its Regulation; Project 1; Identification and Characterization of a Novel Peptide	04/01/93-03/31/97	136,880
	NIH	PO1AR39529 The Osteoclast and its Regulation; Laboratory Core	04/01/93-03/31/97	137,765
	OsteoSA	OsteoSA	01/01/95-12/31/95	2,064,000

O'Connell, P.

Active Support	NIH/NCHGR	1PO1HG00470-05 Project 3. YAC Mapping of STS Markers for Chromosome 3 Project 3	06/01/92-05/31/97	261,626
	NIH/NCHGR	1PO1HG00470-05 Core a. Molecular Biology Core	06/01/92-05/31/97	326,016
	NIH/NCI	2P50CA58183-02 Translational Research in Breast Cancer--San Antonio, Project 4	09/01/95-08/31/99	162,000
	NIH/NCI	R01DK47482-03 Susceptibility Genes in Mexican Americans	09/30/93-09/29/98	216,481
	NIH/NCI	2P01CA55261-01 Molecular and Genetic Epidemiology of Gliomas	01/01/96-12/31/01	81,433
	NIH/NCI	2P30C-541174-03 Molecular Biology Core, San Antonio Institute	08/01/96-07/31/97	64,110
Pending Support	NIH/NCI	5P01CA30195-01 Molecular Oncology Program Project Project 4	03/01/97-02/28/02	101,955

Sharp, Z.D.

Active Support	Aging Research and Education Center	Development of a Mouse Model Using Controlled Expression of Exogenous GH in Pituitary Somatotropes by a Tetracycline Binary Control System	01/01/96-12/31/96	10,544
	NCI	The role of the ATM Protein in Human Breast Cancer	08/01/96-07/31/97	35,000

Tomkinson, A.E.				
Active Support	NIH	R29GM47251-03 Cellular Functions of Eukaryotic DNA Ligases	08/01/93-07/31/98	77,579
	The Council for Tobacco Research	#3786 DNA Nucleotide Excision Repair in Eukaryotes	01/01/94-12/31/96	52,174
	Aging Research and Education Center	Construction of a Transgenic Animal Model to Examine the Involvement of Oxidative DNA Damage and DNA	01/01/96-12/31/96	15,000
Pending Support	March of Dimes	Completion of Meiotic Recombination During Spermatogenesis	07/01/96-06/30/97	80,000
	NIH	Nucleotide Excision Repair in Saccharomyces Cerevisiae	01/01/97-12/31/97	119,191
	American Cancer Society	Nucleotide Excision Repair in Saccharomyces Cerevisiae	01/01/97-12/31/97	240,000
Von Hoff, D.D.				
Active Support	NCI	Phase I Clinical Trials of Anticancer Agents 1-U01 CA69853-02	07/01/95-02/28/98	1,244,228
	NCI	Telomere and Telomerase Interactive Agents 1-U19 CA67760	07/01/95-06/30/00	2,547,539
	NFCR	Intermediates in Gene Amplification	10/01/92-09/30/97	50,000
	USAMRMC	DNA Topoisomerase I-Targeted Therapy for Breast Cancer	06/01/96-05/31/00	600,000
Pending Support	NIH PHS	Blood and Marrow Transplation Cellular and Genetic Therapy; The Importance of Telomerase in the Transplation Setting Therapy for Breast Cancer	07/01/96-06/30/01	466,407 800,000
	NCI	Molecular Studies Task 4 Telomerase Activity in Early Detection of Cancer	09/30/96-09/28/98	386,246

Yee, D.

Active Support	NIH/NCI	K04 CA01670-04 The IGF-System as a Treatment Target in Breast Cancer	09/01/92-08/31/97	64,500
	NIH	P50CA58183-04 SPORE in Breast Cancer, Sub-Project, Career Development Awards in Translational Breast Cancer	09/30/95-07/31/00	144,500
	NIH	P01CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 6; The IGF-System as a Potential Treatment Target in Breast Cancer	07/28/92-02/28/97	131,011
	NIH	PO1CA30195-16 Medical Oncology Program Project; Therapeutic Research; Project 2; Molecular Variants of Estrogen and Progesterone Receptor in Clinical Breast Cancer	07/28/92-02/28/97	116,533
	NIH	R01 CA72621-01 Enhancement of HSV-tk Gene Therapy in Breast Cancer	12/01/96-11/30/01	120,688
Pending Support				
	NIH	5P01 CA30195-17 Markers of Breast Cancer Evolution and Progression, Program Project 6, The IGF-System in Breast Cancer Diagnosis and Treatment	03/01/97-02/28/02	153,027
	NIH	R01, The IGF-System in Breast Cancer Diagnosis and Treatment	04/01/97-03/31/02	161,086

Molecular Medicine Mini Symposium Series

Center for Molecular Medicine / Institute of Biotechnology
University of Texas Health Science Center at San Antonio

Signal Transduction and Cell Cycle

****

- **Dr. Tony Hunter** The Salk Institute 9:30 a.m. - 10:30 a.m.
  Keynote Presentation
  "Cell Cycle Regulation by Protein Phosphorylation "

- *Break* 10:30 a.m. - 11:00 a.m.

- **Dr. Yue Xiong** The University of North Carolina, Chapel Hill 11:00 a.m. - 12:00 noon
  "CDK Inhibitors: Their Function in Cell Cycle Control, Tumor Growth Suppression and Cell Differentiation"

- *Lunch Break* 12:00 noon - 1:00 p.m.

- **Dr. Ted Weinert** The University of Arizona 1:00 p.m. - 2:00 p.m.
  "Yeast Cell Cycle Checkpoints and Lesion Processing Model"

- *Break* 2:00 p.m. - 2:30 p.m.

- **Dr. Paul Russell** The Scripps Research Institute 2:30 p.m. - 3:30 p.m.
  "Stressing the Cell Cycle: MAP Kinase Pathway Links G2/M Control with Cellular Stress Signals "

- *Reception* 3:30 p.m. - 4:30 p.m.

****

Date: Friday, December 1, 1995

Organizer: Eva Lee, Ph.D.

Place: Conference Room 3.002 at the
Institute of Biotechnology
Texas Research Park
15355 Lambda Drive

For information telephone Karen Steger at 210/567-7201

Molecular Medicine Mini Symposium Series

Center for Molecular Medicine/Institute of Biotechnology
University of Texas Health Science Center at San Antonio

DNA Replication, DNA Repair and Genetic Recombination

****

- **Dr. Stephen C. West** Imperial Cancer Research Fund, England — 9:30 a.m. - 10:30 a.m.
  "Protein-directed Molecular Interactions in Genetic Recombination"
- *Break* — 10:30 a.m. - 11:00 a.m.
- **Dr. Thomas A. Kunkel** NIEHS, Research Triangle Park, NC — 11:00 a.m. - 12:00 a.m.
  "DNA Replication Fidelity, Mismatch Repair and Genome Stability"
- *Lunch Break* — 12:00 p.m. - 1:30 p.m.
- **Dr. Philip C. Hanawalt** Stanford University, Stanford, CA — 1:30 p.m. - 2:30 p.m.
  "Mechanisms and Consequences of Transcription-Coupled DNA Repair"
- *Break* — 2:30 p.m. - 3:00 p.m.
- **Dr. Errol C. Friedberg** Southwestern Medical Center, Dallas, TX — 3:00 p.m. - 4:00 p.m.
  "Human Hereditary Diseases With Defective NER: New Answers and New Questions"
- *Reception* — 4:00 p.m. - 5:00 p.m.

****

Thursday, February 8, 1996

Organizer: Alan Tomkinson
Place: Conference Room 3.002 at the
Institute of Biotechnology
Texas Research Park
15355 Lambda Drive
For information telephone Karen Steger at 210/567-7201

Molecular Medicine Mini Symposium Series

Center for Molecular Medicine/Institute of Biotechnology
University of Texas Health Science Center at San Antonio

Molecular Genetics of Cancer

****

9:00 - 9:10 a.m.	*Welcoming Remarks* **Dr. Wen-Hwa Lee, Professor and Director, Institute of Biotechnology**
9:10 - 10:10 a.m.	*"Chromosomes, Genes, and Human Cancer"* **Dr. Peter C. Nowell, Univ. of Pennsylvania School of Medicine**
10:10 - 10:30 a.m.	Break
10:30 - 11:30 a.m.	*"The Regulation of p53 Function"* **Dr. Gigi Lozano, MD Anderson Cancer Center, Houston, TX**
11:30 - 1:00 p.m.	Lunch
1:00 - 2:00 p.m.	*"Molecular Mechanisms of Human Papillomavirus - Associated Neoplasias"* **Dr. Louise T. Chow, University of Alabama, Birmingham, AL**
2:00 - 2:30 p.m.	Break
2:30 - 3:30 p.m.	*"Ras Oncogenes and Signal Transduction Pathways"* **Dr. Geoffrey Cooper, Dana-Farber Cancer Institute, Boston, MA**
3:30 - 4:30 p.m.	Reception

****

Friday, April 5, 1996

Organizer: Ed Seto

1996 PORT ARANSAS RESEARCH CONFERENCE AGENDA

FRIDAY, AUGUST 16

7:00 p.m. — Opening Remarks, Wen-Hwa Lee, Ph.D.
Introduction of New Graduate Students

7:15 p.m. - 10:00 p.m. — Poster Session/Social Mixer

POSTER PRESENTATIONS

Gang Chen, Ph.D. (Presented by Eva Lee, Ph.D.)
"Characterization of the Protein Product of ATM Gene that is Critical in Cell Cycle Checkpoints and DNA Recombination"

Phang-Lang Chen, Ph.D.
"Retinoblastoma Protein Positively Regulates Terminal Adipocyte Differentiation through Interaction with C/EBPS"

Kathryn Fischbach, Ph.D.
"Search for a Metastisis Gene on Chromosome 14"

Wayne Hao
"Phosphoinositides as Regulators of Clathrin Assembly"

Adrian Lee, Ph.D.
"*In Vitro* and *In Vivo* Inducible Gene Expression in Human Breast Cancer Cells"

Pierre Lemieux, Ph.D.
"Overexpression of the Small Heat Shock Protein Hsp27 Increases Invasive Properties of Breast Cancer Cells"

Jackie Lin
"A Multiple Step Tumorigenesis in Mouse"

Zachary Mackey

"Mammalian DNA Ligases"

Irena Melnikova

"Functional Domains of Helix-Loop-Helix Id3 Protein"

Alexander Nikiten, M.D.,Ph.D./Daniel J. Riley, M.D.

"Adenovirus-mediated *Retinoblastoma* Gene Therapy Suppressess Spontaneous Pituitary Melanotroph Tumore in $Rb^{+/-}$ Mice"

Steve Townson, Ph.D.

"Honeybee Opsins"

James Wang

"Characterization of Proteins Binding to the ZII Element in the Epstein-Barr Virus BZLF1 Promoter: Transactivation by ATF1"

Limin Yang, M.D., Ph.D.

"Retinoids Inhibit Cell Proliferation and AP-1 Activity in Human Breast Cells"

Qin Zhang

"Cloning and Expression of a Suppressor of the BZLF1 Promoter"

SATURDAY, AUGUST 17

7:30 a.m.	Continental Breakfast

FACULTY PRESENTATIONS

8:00 a.m. - 8:40 a.m.	"Maintenance of Genetic Stability in Eukaryotes" ***Alan Tomkinson, Ph.D.*** Institute of Biotechnology
8:40 a.m. - 9:20 a.m.	"Cell Cycle Control and DNA Repair in Tumor Predisposition" ***Eva Lee, Ph.D.*** Institute of Biotechnology
9:20 a.m. - 10:00 a.m.	"Tumor Suppressor Genes of Breast Cancer: BRCA1 and BRCA2" ***Wen-Hwa Lee, Ph.D.*** Institute of Biotechnology
10:00 a.m. - 10:15 a.m.	Break
10:15 a.m. - 10:55 a.m.	"Mechanisms of Tamoxifen Resistance in Breast Cancer" ***C. Kent Osborne, M.D.*** Department of Medicine
10:55 a.m. - 11:35 p.m.	"Regulation of Breast Cancer Growth by Insulin-like Growth Factors" ***Douglas Yee, M.D.*** Department of Medicine
11:35 p.m. - 12:15 p.m.	"Estrogen Receptor Variants in Breast Cancer" ***Suzanne Fuqua, Ph.D.*** Department of Medicine
12:15 p.m. - 3:00 p.m.	Lunch Break

3:00 p.m. - 3:40 p.m.	"Molecular Genetic Studies of Breast Cancer Evolution" ***Peter O'Connell, Ph.D./Craig Allred, M.D.*** Department of Pathology
3:40 p.m. - 4:20 p.m.	"Role of Specific Transcription Factors in Breast Tumorigenesis" ***Powel H. Brown, M.D., Ph.D.*** Department of Medicine
4:20 p.m. - 5:00 p.m.	"BRCA2 & Breast Cancer" ***Z. Dave Sharp, Ph.D.*** Institute of Biotechnology
5:00 p.m. - 5:40 p.m.	"Id Proteins in Growth and Development" ***Barbara A. Christy, Ph.D.*** Institute of Biotechnology
5:40 p.m.	Adjourn for evening

SUNDAY, AUGUST 18

7:30 a.m.	Continental Breakfast

FACULTY PRESENTATIONS

8:00 a.m. - 8:40 a.m.	"Regulation of Ion Channel Gene Expression in the Brain" ***Paul D. Gardner, Ph.D.*** Institute of Biotechnology
8:40 a.m. - 9:20 a.m.	"Vesicle Trafficking in Nerve Terminals" ***Eileen M. Lafer, Ph.D.*** Institute of Biotechnology
9:20 a.m. - 10:00 a.m.	"Vision in Drosophila: Color Sensitivity and Photoreceptor Differentiation" ***Steven G. Britt, M.D.*** Institute of Biotechnology

1996 Port Aransas Research Conference
August 16-18, 1996

Molecular Medicine Student Attendees

Mr. Jerry Bates
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7201

Ms. Chi-Fen Chen
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7201

Ms. Ling Chen
Molecular Medicine Graduate
Institute of Biotechnology
UT Ext. 7333

Mr. Wen-Hai Chou
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7255

Ms. Linda deGraffenried
Molecular Medicine Graduate Student
Department of Medicine
UT Ext. 4790

Ms. Jill Gilroy
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7201

Ms. Jennifer Gooch
Molecular Medicine Graduate Student
Department of Medicine
UT Ext. 6674

Mr. Wayne Hao
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7280

Ms. Pei-Fen Hsin
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7201

Mr. David Levin
Molecular Medicine Graduate Student
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UT Ext. 7333

Ms. Jackie Lin
Molecular Medicine Graduate Student
Institute of Biotechnology
UT Ext. 7332

Ms. Qun Liu
Molecular Medicine Graduate Student
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Mr. Zheng Luo
Molecular Medicine Graduate Student
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Mr. Zachary Mackey
Molecular Medicine Graduate Student
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Ms. Michelle Martin
Molecular Medicine Graduate Student
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Ms. Irena Melnikova
Molecular Medicine Graduate Student
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Mr. Jonathan Mlocek
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Ms. Ashby Morrison
Molecular Medicine Graduate Student
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Mr. Hongyi Pan
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Mr. William Ramos
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Mr. Ernesto Salcedo
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Mr. Binwei Song
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Mr. Kelly Trujillo
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Ms. Yi-Chinn Ueng
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Mr. Ahmad Utomo
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Mr. James Wang
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Mr. Eric Wooten
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Mr. Qing Zhong
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Inactivation of the mouse *Brca1* gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development

Chia-Yang Liu, Andrea Flesken-Nikitin, Shang Li, Yingying Zeng, and Wen-Hwa Lee[1]

Center for Molecular Medicine/Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245 USA

***BRCA1* is proposed to be a tumor suppressor gene. To explore the biological function of *BRCA1*, a partial deletion (amino acids 300–361) of mouse *Brca1* exon 11 was introduced into the genome of embryonic stem (ES) cells by homologous recombination. Mice carrying one mutated allele of *Brca1* appear normal and are fertile up to 10 months of age without any sign of illness. However, no viable progeny homozygous for the *Brca1* mutant allele were obtained. Detailed analysis of large numbers of embryos at different stages of development indicated that the homozygous mutant concepti are severely retarded in growth as early as embryonic day 4.5 (E4.5) and are resorbed completely by E8.5. Although the homozygotes at E5.5–E6.5 are able to synthesize DNA and display distinguishable embryonic and extraembryonic structures, they fail to differentiate and form egg cylinders. Consequently, they were unable to form primitive streaks and undergo gastrulation. Consistent with these in vivo results, blastocysts homozygous for mutated *Brca1* alleles are at a considerable disadvantage when grown in vitro. These observations suggest that *Brca1* has an important role in the early development of mouse embryos.**

[*Key Words*: *Brca1* gene; gene targeting; mouse embryogenesis]

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Hereditary breast cancer is characterized as an early-onset, bilateral disease and is associated with other tumors of ovarian, endometrial, and prostate origins (Tulinius et al. 1992; Anderson and Badzioch 1993; Sellers et al. 1994). The heterogeneity in breast cancer suggests that the manifestation of multiple genetic factors intertwines with environmental factors, such as hormones and diet, that modify expression of the phenotype. At the molecular level, breast tumor development is thought to result from mutations of several growth regulatory genes. The *BRCA1* gene is mutated in the germ line of a subset of families with inherited breast cancer, and loss of the remaining wild-type allele is often found in tumor tissues (Hall et al. 1990; Smith et al. 1992; Miki et al. 1994). This recessive nature of *BRCA1* fits with the properties of human tumor suppressor genes (Riley et al. 1994).

BRCA1 mutations are associated with 45% of familial breast cancer, which accounts for ~10% of the total number of these neoplasms. However, they are rarely linked with sporadic cases (Futreal et al. 1994). Therefore, the role of *BRCA1* in the pathogenesis of 95% of breast cancer is unclear. Interestingly, the *BRCA1* gene product, which is normally a nuclear protein, is aberrantly mislocated in the cytoplasm of most breast tumor cell lines (Chen et al. 1995). Although the molecular mechanism for the failure of nuclear transport is not known, these findings suggest that BRCA1 may be involved in many breast cancers, sporadic as well as familial.

BRCA1 is a large gene spread over ~100 kb of the genome. It consists of 24 exons and encodes a nuclear phosphoprotein of 1863 amino acids with a molecular weight of ~220 kD (Chen et al. 1995, 1996). BRCA1 is a novel protein with an amino-terminal ring finger motif similar to those found in other proteins that interact with DNA, RNA, or proteins (Miki et al. 1994). The mouse *BRCA1* homolog, *Brca1*, was characterized recently. The predicted *Brca1* gene product shares ~58% amino-acid identity with the human protein, and the mRNA expression patterns in mice and humans are also quite similar. Interestingly, *Brca1* mRNA expression in mouse mammary gland involution parallels its differentiation, suggesting an important regulatory role for Brca1 in tissue proliferation and differentiation (Lane et al. 1995; Marquis et al. 1995).

To understand the physiological function of *Brca1* and study its role in breast and ovarian carcinogenesis, we

[1]Corresponding author.

established mutant *Brca1*(+/−) mouse lines using gene targeting in embryonic stem (ES) cells. Mice lacking one wild-type allele of the *Brca1* appear normal and are fertile until 10 months of age. However, *Brca1*(−/−) mutants die during early embryogenesis. Histological examinations of early concepti and genotyping after fixation by microdissection–polymerase chain reaction (MD–PCR) demonstrated that *Brca1*(−/−) mutant embryos are growth-retarded as early as embryonic day (E4.5). They form embryonic and extraembryonic tissues but develop into severely disorganized masses incapable of forming egg cylinders at E5.5–E6.5. The embryos are resorbed completely by E8.5. These results reveal an important role for the *Brca1* gene in early mouse development.

Results

Targeted mutation of the mouse BRCA1 *homolog*

For genetic ablation of mouse *Brca1*, exon 11 was chosen as the target because its counterpart in the human *BRCA1* is the largest coding exon (3425 bp) and is frequently mutated in families with histories of breast and ovarian cancer. Mouse *Brca1* was obtained by screening a 129/Sv-derived genomic library with PCR-amplified DNA from exon 11 of the human *BRCA1*.

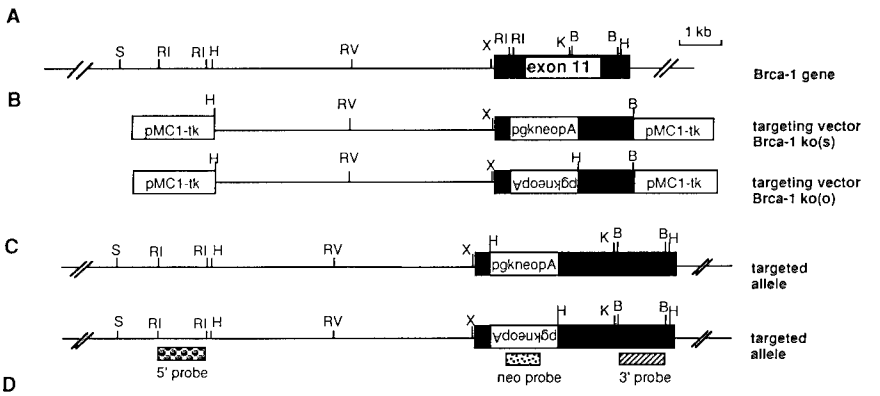
The targeting vector was constructed by deleting a 184-bp *Eco*RI fragment corresponding to mouse Brca1 amino acids 300–361 (Fig. 1A) and replacing it with a *pgkneopA* cassette in either the sense (s) or antisense (o) orientation. These two constructs were then subcloned into the p2TK vector to produce two final targeting vectors designated as Brca1–ko(s) and Brca1–ko(o), respectively (Fig. 1B). Both vectors were individually transfected into ES cells, and 384 colonies doubly resistant to G418 and FIAU were isolated. DNA from these colonies was analyzed by Southern blotting to identify clones containing a disruption of the *Brca1* gene resulting from a targeted homologous recombination event. These were identified by the appearance of a novel *Hin*dIII fragment of the predicted size using a fragment of *Brca1* genomic DNA lying 3′ external to the targeting vector as a hybridization probe (Figs. 1C and 2A). The use of Brca1-ko(s) and Brca1-ko(o) vectors resulted in a total of 6 and 13 homologous recombinant clones, respectively. Brca1–ko(o) #3 and Brca1–ko(s) #291 were analyzed further by Southern blot analysis using different probes to confirm the targeted gene disruption (Fig. 2B).

Production of Brca1 heterozygote mutants by germ-line transmission

ES cells from clone Brca1–ko(o) #3 and Brca1–ko(s) #291 were injected independently into C57BL/6J blastocysts, which were then implanted into the uteri of pseudopregnant CBA female foster mice. We generated 5 germ-line chimeras from a total of 16 male chimeras, all derived from clone Brca1–ko(o) #3. Of the 120 offspring from male chimera/female C57BL/6J crosses, 53% were heterozygous (*Brca1*+/−) and 47% homozygous wild type (*Brca1*+/+), as shown by PCR analysis of toe DNA samples. The heterozygous mice were further confirmed by Southern blot analysis of tail DNA samples (data not shown). Heterozygous animals appear normal, healthy, and are fertile for at least 10 months after birth.

Absence of wild-type Brca1 alleles leads to embryonic lethality

To investigate the in vivo effect of the homozygous mutations, *Brca1*(+/−) mice were interbred. The genotypes of offspring were determined at 1 week of age by PCR



	5′ probe	3′ probe	neo probe	
	SalI + BamHI	HindIII	SalI + BamHI	HindIII
Endogenous	11.0 kb	10.0 kb	-	-
Recombinant	12.4 kb	4.6 kb or 3.0 kb	12.4 kb	4.6 kb or 8.0 kb

Figure 1. Strategy for the generation of a targeted mutation in the mouse *Brca1* gene. (*A*) Restriction map of the mouse *Brca1* fragment, encompassing exon 11 and flanking DNA. An 8.0-kb *Hin*dIII–*Bam*HI fragment was used to create the replacement targeting vector. (*B*) Restriction map of the targeting constructs Brca1–ko(s) and Brca1–ko(o). A 184-bp *Eco*RI fragment from the 5′ end of exon 11 was deleted and replaced with a *pgkneopA* cassette in the sense and antisense orientation with respect to the *Brca1* gene. In addition, the genomic fragment was flanked by two pMC1-tk cassettes. (*C*) The predicted structure of a mutant allele after homologous recombination. The probes used for identification of allele-specific recombination are also shown. (*D*) The expected sizes of various restriction fragments detected by flanking and *neo* probes. Abbreviations: (RI) *Eco*RI; (RV) *Eco*RV; (B) *Bam*HI; (H) *Hin*dIII; (S) *Sal*I; (X) *Xho*I.

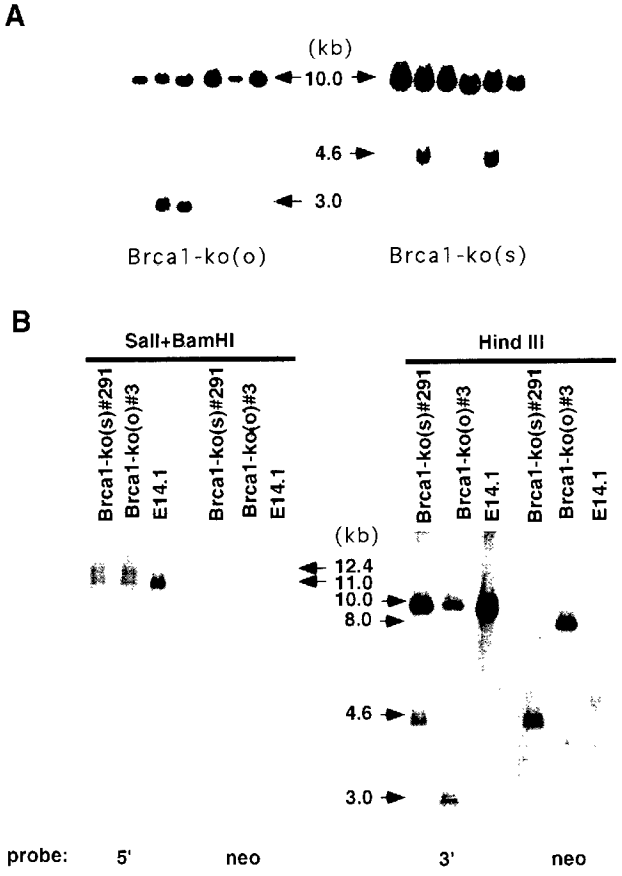


Figure 2. Targeted disruption of the *Brca1* gene in mouse ES cells. (*A*) Initial screen of the targeted *Brca1* clones by Southern blotting analysis. The *Hin*dIII 4.6- or 3.0-kb restriction fragment indicated the expected recombinant allele by Brca1–ko(s) or Brca1–ko(o) vectors, respectively. (*B*) Confirmation of the targeted disruption. DNA samples from parental E14.1 cells and two candidate recombinant clones [Brca1–ko(o) 3 and Brca1–ko(s) 291] were digested with *Hin*dIII or *Sal*I–*Bam*HI and probed with either of the flanking probes or the *neo* probe. An additional fragment of the expected size was found in each of the recombinant clones.

analysis of toe DNA samples. Of the 97 animals tested, 63% were heterozygous and 37% were wild type. No mice homozygous for the mutation were detected, indicating that the *Brca1*(−/−) mutants died in utero (Table 1). To determine the time of death, pregnant females from *Brca1*(+/−) intercrosses were sacrificed, and the fetuses at different gestation times from E8.5 to E12.5 were examined. Of the 81 decidua tested, 58 (72%) contained morphologically normal fetuses, of which 39 (48%) were heterozygous, 19 (24%) were wild type, and none were homozygous. The remaining 23 (28%) decidua were much smaller and contained completely resorbed embryos (Table 1; Fig. 3J,O), indicating that the *Brca1*(−/−) mutant concepti die before E8.5.

Developmental deficiency in Brca1(−/−) *mutant embryos appears shortly after postimplantation*

To precisely pinpoint the differences between wild-type and *Brca1*(−/−) mutant embryos, we next examined the histology of embryos between implantation and gastrulation. Intact decidual swellings of litters from *Brca1*(+/−) intercrosses obtained between E4.5 and E7.5 (Table 1) were fixed, sectioned, and stained with hematoxylin/eosin. Following implantation (E4.5–E5.5), abnormalities that distinguished normal concepti from *Brca1*(−/−) concepti could be readily observed. Both wild-type (Fig. 3A,B) and heterozygous embryos (data not shown) show normal growth and elongation of the egg cylinder, which contains both embryonic and extraembryonic ectoderm and distinct proamniotic cavities. In striking contrast, *Brca1*(−/−) embryos are at least 50% smaller than the wild type and fail to form egg cylinders (Fig. 3F,G,K,L), although they do display embryonic and extraembryonic tissues at this stage (Fig. 3G,L). By E6.5, wild-type embryos are almost ready for gastrulation, with the egg cylinders nearly filling the yolk sac cavities. Elongated proamniotic and distinct exocoelomic cavities are also well developed (Fig. 3C). By comparison, *Brca1*(−/−) embryos show increasing cellular disorganization and start to degenerate (Fig. 3H,M). By the time wild-type embryos undergo gastrulation (E7.5) and the mesoderm develops (Fig. 3D), the *Brca1*(−/−) embryos are significantly developmentally retarded and there is no sign of mesoderm differentiation (Fig. 3I,N). Of the 26 decidua examined at E8.5, 8 (30%) contained no embryo, indicating complete resorption (Table 1; Fig. 3J,O).

Table 1. *Genotype analysis of the progeny from* Brca1(+/−) *heterozygous intercrosses*

Age (DNA source)	Litter	Number	Genotype			Resorbed
			+/+	+/−	−/−	
10 days (toes)	19	97	36	61	0	
E9.5–12.5 (yolk sac)	7	55	11	29	0	15
E8.5 (yolk sac)	1	9	3	5	0	1
E8.5 (paraffin sections)	2	17	5	5	0	7
E7.5 (paraffin sections)	3	23	6	14	3	
E6.5 (paraffin sections)	5	37	10	20	7	
E5.5 (paraffin sections)	3	21	10	6	5	
E4.5 (paraffin sections)	1	7	3	2	2	
E3.5 (outgrowth)	6	37	10	19	8	

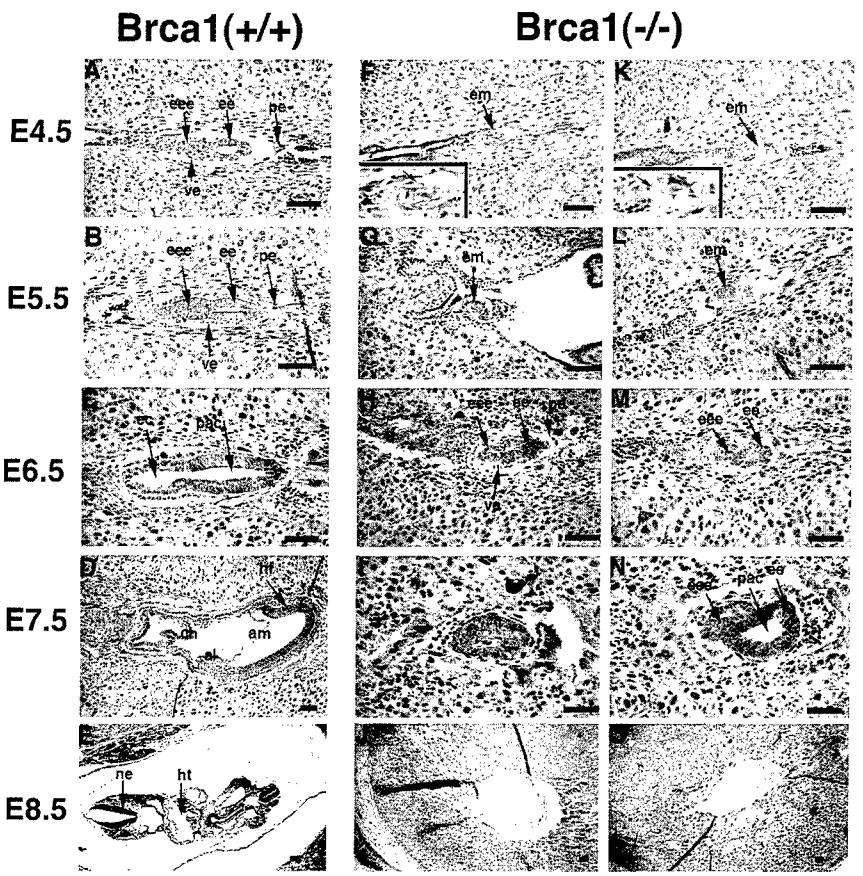


Figure 3. Histological sections of wild-type and *Brca1*(−/−) mutant embryos grown in utero. The uteri of *Brca1*(+/−) females were dissected between 4.5 and 8.5 days after intercross matings, and 4-μm sections were prepared as described in Materials and methods. All uterine decidua were sectioned transversely according to the nomenclature of Smith (1985), and the mesometrial to anti-mesometrial axis is *left* to *right*. (*A–E*) Wild-type embryos; (*F–O*) *Brca1*(−/−) embryos; (*A,F,K*) E4.5 egg cylinders; (*B,G,L*) E5.5 egg cylinders; (*C,H,M*) E6.5 embryos; (*D,I,N*) E7.5 embryos; (*E,J,O*) E8.5 embryos. Note the appearance of the proamniotic cavity and the clearly differentiated embryonic and extraembryonic ectoderm in a wild-type E4.5 egg cylinder (*A*), an elongated egg cylinder ready for gastrulation in E6.5 embryo (*C*), and a gastrulating embryo with primitive streak and mesoderm differentiation in an E7.5 embryo (*D*). Abbreviations: (eee) Extra-embryonic ectoderm; (pac) proamniotic cavity; (al) allantois; (am) amnion; (ch) chorion; (ec) exocoelomic cavity; (ee) embryonic ectoderm; (em) embryo mass; (hf) head fold; (ht) heart; (ne) neuroepithelium; (pe) parietal endoderm; (ve) viceral endoderm. (*Insets* in *F* and *K*) enlarged twofold. Bar, 100 μm.

The morphologically abnormal phenotype is inferred to be the consequence of the homozygous mutant genotype. To confirm that the smaller malformed embryos do result from the loss of the wild-type *Brca1* gene, sectioned embryonic tissues are collected by microdissection and subjected to PCR genotyping analysis. PCR analysis of E4.5 littermates representative of *Brca1* heterozygote matings shows that the developmentally impaired embryos are homozygous for the mutated *Brca1* allele (Fig. 4A, right, and B). These observations demonstrate that *Brca1*(−/−) embryos have growth and morphogenetic defects before the onset of gastrulation and die before E8.5. To test whether the *Brca1*(−/−) embryos between E5.5 and E6.5 remain alive, we injected 5-bromo-2′-deoxyuridine (BrdU) (100 μg/gram body weight) intraperitoneally into heterozygous pregnant females 1 hr before sacrifice. The embryos were fixed, sectioned, and subjected to immunostaining with anti-BrdU antibody. The *Brca1*(−/−) embryos were found to incorporate BrdU, indicating that the mutant embryos synthesize DNA (Fig. 5C,D). These results suggest that the cells are still alive, although the embryos are underdeveloped. When we compared the percentage of BrdU-labeled cells of three genotypes (+/+, +/−, and −/−) at E5.5–E6.5 (Fig. 5E), the wild-type and heterozygote embryos had ~82%–85% of their nuclei labeled, whereas the homozygous mutant embryos had only 61%–63%. On the other hand, when we compared apoptotic index obtained by TUNEL [terminal deoxynucleotide transferase TdT)-mediated dUTP-biotin nick end labeling] assays of wild-type and mutant embryos, no significant difference was found (data not shown). These results indicate that the overall growth and morphogenetic defect in the *Brca1*(−/−) mutant embryos are, at least in part, attributable to a decrease in the proliferation capability, not an increase in apoptosis of embryonic cells.

Brca1(−/−) *blastocysts have a growth disadvantage in culture*

Blastocyst outgrowth in an in vitro culture offers an alternative method to study early postimplantational development. Blastocysts (E3.5) from intercrosses between *Brca1*(+/−) mice were isolated by uterine flushing, and photographed individually before and after in vitro culture. All 37 blastocysts examined were indistinguishable (Fig. 6A,C,E,H), indicating that embryos homozygous for the targeted mutation of *Brca1* were morphologically normal before implantation. However, a disadvantage in the outgrowth of homozygous embryos was noted after 5 days in culture. Whereas cultured blastocysts of each genotype (+/+, +/−, and −/−) gave rise to adherent sheets of trophoblastic giant cells, the *Brca1*(−/−) blastocysts showed impaired outgrowth of the inner cell mass (Fig. 6F,G,I,J). These results are consistent with the

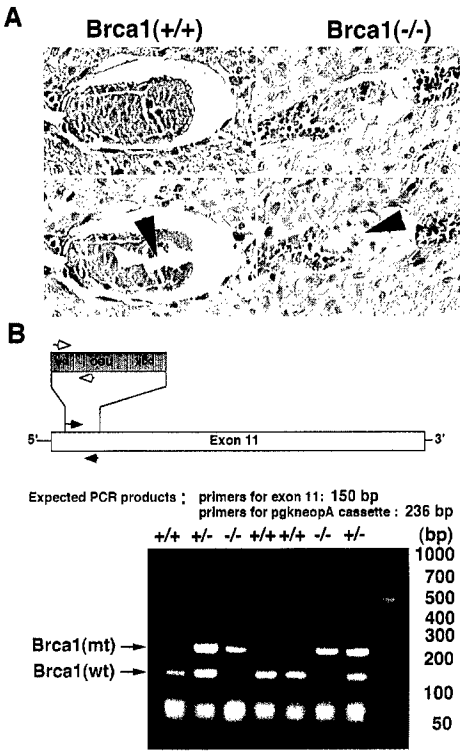


Figure 4. MD–PCR genotype analysis of littermate E4.5 embryos from a *Brca1* heterozygote intercross. (*A*) Representative images before (*top*) and after (*bottom*) microdissection. Arrowheads point to the area from which the embryo's cells had been dissected out for DNA isolation and PCR analysis. (*B*) Design of primers for detection of exon 11 in the wild-type *Brca1* gene (solid arrow), and those for the *pgkneopA* cassette (open arrow) integrated into mouse genome by homologous recombination. PCR products amplified from the DNA of microdissected embryonic samples were resolved on a 4% NuSieve/SeaKem (3:1) agarose gel in TAE buffer. Abbreviations: (pgk) phosphoglycerate kinase I promoter; (neo) gene for neomycin resistance; (pA) polyadenylation sequence of the bovine growth hormone gene.

in vivo observations of growth retardation in homozygous embryos as described above.

Discussion

In humans, mutations of the *BRCA1* gene are strongly implicated in familial breast, ovarian, and perhaps other types of cancer. One of the purposes in generating *Brca1* mutant mice was to establish an in vivo model system to study the genetic and/or environmental factors responsible for the pathogenesis of these cancers. In concert with Knudson's "two-hit" theory of carcinogenesis (Knudson 1971), germ-line mutation of one allele of the *BRCA1* gene followed by loss of the other allele in somatic cells is currently a favored explanation for familial breast and ovarian tumorigenesis. If human *BRCA1* and mouse *Brca1* are functionally equivalent, *Brca1*(+/−) mice should produce breast, ovarian, and/or other type of cancers. Consistent with the results published previously (Gowen et al. 1996), neither virgin nor naturally mated female *Brca1*(+/−) mice, up to 10 months of age, showed any tumors or illness. However, we cannot rule out at this time the possibility of a predisposition for tumorigenesis in older animals.

The finding of one woman homozygous for the *BRCA1* mutation (Boyd et al. 1995) led to the notion that *BRCA1* is dispensable for human development and to the expectation that *Brca1*(−/−) mutant mice would be viable. In contrast, the published and the current results demonstrated that the *Brca1* gene has a crucial role during embryonic development in mice, indicating either that an absence of *BRCA1* has different consequences in humans and mice or that the different mutations generated in this large protein—in mice experimentally and in humans naturally—have dissected separate functions important for development and tumorigenesis.

Brca1(−/−) blastocysts appear to be normal. They hatch, invade the uterine epithelium, and attach to the basement membrane that separates the uterine stroma and epithelium. In our experiments, the *Brca1*(−/−) mutant phenotype is not apparent until after completion

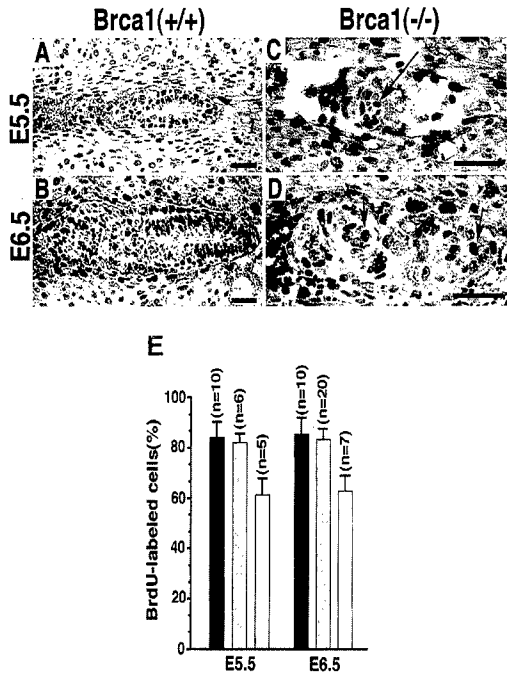


Figure 5. Epiblast cells proliferation is retarded in *Brca1*(−/−) mutant embryos. In vivo BrdU incorporation was described in Materials and methods. (*A–D*) Transverse sections of uterine decidua were immunostained with an anti-BrdU monoclonal antibody. (*A,B*) Wild-type embryos; (*C,D*) *Brca1*(−/−) embryos; (*A,C*) E5.5 egg cylinders; (*B,D*) E6.5 egg cylinders. Arrows point to BrdU-labeled cells (dark brown). Unlabeled nuclei appear blue as a result of counterstaining with hematoxylin. (*E*) Histogram summary of the percentage of BrdU-labeled cells of different genotypes. (Solid bar) *Brca1*(+/+); (shaded bar) *Brca1*(+/−); (open bar)*Brca1*(−/−). Numbers in parenthesis indicate the number of embryos analyzed. Two-tailed *P* value (homozygote mutant vs. wild-type/heterozygote) is <0.0001. Bar, 50 μm.

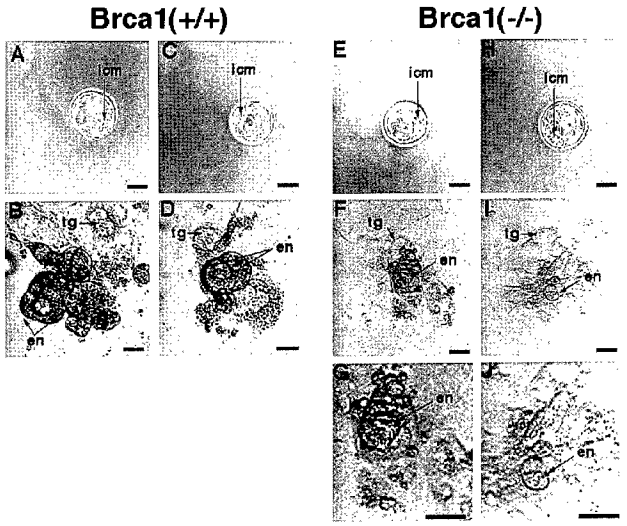


Figure 6. Outgrowths of wild-type and *Brca1*(−/−) blastocysts in vitro. Wild-type blastocysts (*A*,*C*) and *Brca1*(−/−) blastocysts (*E*,*H*) appear to be morphologically normal (arrows point to inner cell mass). Representative example of wild-type (*B*,*D*) and *Brca1*(−/−) (*F*,*I*) blastocysts cultured for 5 days in vitro. *G* and *J* are higher magnifications of *F* and *I*, respectively. Abbreviations: (en) endoderm; (icm) inner cell mass; (tg) trophoblast giant cells. Bar, 10 μm.

of these steps in implantation. After implantation (E4.5), but before gastrulation (E7.5), the inner cell mass of wild-type embryos undergoes rapid proliferation that extends into the blastocoel cavity to form a structure known as the egg cylinder (Fig. 3A–C). The egg cylinder is initially a double-layered structure that encloses a narrow lumen termed the proamniotic cavity. The two layers consist of an inner layer of ectoderm and an outer layer of endoderm cells (Kaufman 1992). In *Brca1*(−/−) mutant embryos, the embryonic and extraembryonic tissues are present but are severely retarded in growth. Although the cells remain alive, the embryos are incapable of proceeding toward gastrulation (Fig. 3F–I,K–N). By E8.5, the *Brca1*(−/−) concepti are resorbed completely. Because maternal RNAs are typically degraded (Sawicki et al. 1981) and at least some embryonic de novo gene expression is required (Johnson 1981) after implantation, it is not clear whether maternal Brca1 can maintain the cell viability until E7.5. Consistent with in vivo observations, the in vitro blastocyst outgrowth experiments also showed a disadvantage in the growth of the *Brca1*(−/−) inner cell mass (Fig. 6), suggesting that the *Brca1* is important for cell growth at this early embryonic stage.

The phenotype of *Brca1*(−/−) embryos described here is similar to those of other mutant mice. The murine *evx1*(−/−) mutant leads to embryonic malformations after implantation but before gastrulation (Spyropoulos and Capecchi 1994). Unlike *Brca1*(−/−) mutants, *evx1*(−/−) mutants failed to differentiate into distinct embryonic and extraembryonic tissues. Cells in *evx1*(−/−) embryos do not establish proper communication between embryonic and extraembryonic tissues, which is critical for their further differentiation. The *fgfr-1*(−/−) mouse mutant is also similar in terms of the timing of embryonic demise. Unlike *Brca1*(−/−) mutants, however, *fgfr-1*(−/−) concepti do form egg cylinders, undergo gastrulation, and generate mesoderm. In this instance, embryonic lethality may be caused by the aberrant mesodermal patterning (Deng et al. 1994; Yamaguchi et al. 1994). The embryos of the *fug1* (failure to undergo gastrulation) mouse mutant seem to be the closest to the *Brca1*(−/−) homozygotes with regard to timing and the high degree of disorganization within the embryo. Homozygous *fug1* mutant embryos also arrest growth at the egg cylinder stage (E6.0) and are mostly resorbed by E8.5 (DeGregori et al. 1994). Little is known about the genes required for the differentiation of primitive ectoderm and endoderm, the formation of the proamniotic cavity, or the organization of the ectoderm into an organized epithelium. The genetic evidence from our results demonstrated that like *fug1*, *Brca1* may be required for the organized development of the embryo at the egg cylinder stage, or for subsequent gastrulation. Whether *Brca1* interacts with *fug1* or other cellular factors in the control of early morphogenesis is unknown.

The developmental stage of embryonic death of the *Brca1*(−/−) mutant in our experiments was different from that published by Gowen et al. (1996). In their report, mice lacking a functional *Brca1* gene exhibited neural tube defects at E9.5. These neural defects were proposed to be the major cause of embryonic lethality between E10 and E13, which is later than what we observed here. To confirm that the mutant phenotype was concurrent with loss of the wild-type *Brca1* gene, we genotyped all sectioned decidua by MD–PCR and obtained a complete correlation between the mutant genotype and the phenotype. Although the discrepancy between these two observations remains to be resolved, the phenotypic variation of the two *Brca1* mutations is probably not attributable to different genetic backgrounds because both mutations were analyzed on the hybrid 129/C57BL backgrounds.

Interestingly, the phenotypes of three *MRF4* homozygous mutants varied from perinatal death to viable with only minor abnormalities (Braun and Arnold 1995; Patapoutian et al. 1995; Zhang et al. 1995). One potential explanation for these phenotypic variations is that the regulation of a neighboring gene, named *Myf5*, was interfered with by two of the constructs but not by the other (Olson et al. 1996). In our targeting construct, a 184-bp *Eco*RI fragment within the 5′ end of exon 11 was deleted and replaced with the *pgkneopA* cassette. In their construct, Gowen et al. (1996) deleted a 1.7-kb *Xho*I–*Kpn*I genomic fragment that contains 0.1 kb of intron 10 in addition to 1.6 kb of exon 11. Although we do not know whether either of the insertion/deletion manipulations of *Brca1* affects the expression of other genes surrounding exon 11 of *Brca1*, removal of a splicing acceptor site in the latter construct may generate different splicing variants of *Brca1*. Nevertheless, the evidence together strongly supports the notion that *Brca1* is needed for mouse early embryonic development.

Recently, we isolated two Brca1-interacting proteins that are structurally similar to transcription factors. One is a LIM-type homeo box-containing protein and the other is a zinc-finger motif-containing protein (S. Li and W.-H. Lee, unpubl.). Both classes of proteins are known to participate in the control of development. Our hypothesis is that Brca1, at least in the mouse, somehow participates in regulating the spatial–temporal organization of the developing embryo through interactions with these, and perhaps other, developmental control proteins. This exciting possibility is currently under investigation.

Materials and methods

Construction of targeting vectors

The mouse *Brca1* gene was isolated by screening a λDASH mouse genomic library derived from the 129/Sv mouse strain (provided by Dr. Tom Doetschman, University of Cincinnati, OH), using a 3.5-kb fragment of human *BRCA1* exon 11 as a probe. Positive clones were subcloned into the pBluescript SK vector (Stratagene). Restriction mapping and DNA sequencing of the intron 10–exon 11 junction yielded the restriction map shown in Figure 1A. To generate a targeting vector, an 8.0-kb *Hin*dIII–*Bam*HI fragment of the mouse *Brca1* gene containing exon 11 was subcloned into the pBluescript SK vector (Stratagene), yielding the plasmid pHB-8. The *Hin*dIII site was then opened and changed to *Bam*HI by ligation with a *Bam*HI linker. A 184-bp *Eco*RI fragment, corresponding to amino acid residues 300–361, was deleted and replaced with a *pgkneopA* cassette (Soriano et al. 1991) in both the sense and antisense orientation [designated as Brca1–neo(s) and Brca1–neo(o), respectively]. These two constructs were then subcloned into the p2TK vector (Lee et al. 1992) to produce two final targeting vectors designated as Brca1–ko(s) and Brca1–ko(o), respectively.

Electroporation and selection of ES cells

E14.1 ES (Handyside et al. 1989) cells derived from mouse strain 129/Ola were maintained on a monolayer of mitomycin C-inactivated, neomycin-resistant, fibroblast feeder cells, as described previously (Robertson 1987). Thirty micrograms of *Sal*I-linearized targeting vector was electroporated into a suspension of trypsinized cells (7×10^6) in Dulbecco's modified Eagle medium (DMEM) (GIBCO/BRL) using a Bio-Rad gene pulser (250 μF, 800 V). Cells were then incubated at room temperature for 5 min, plated, and allowed to recover for 24 hr before selection in medium containing G418 (250 μg/ml) and FIAU (1 μM). Cells were fed daily, and after 8 days the resulting double-resistant ES clones were individually picked and transferred onto 24-well plates with feeders. On the following day, each clone was trypsinized and divided in half. One half was frozen (−80°C), whereas the other half was plated into a 12-well plate without feeder cells and used to prepare DNA.

Analysis of targeted ES cell clones

The analysis of DNA from ES cell clones was described previously (Laird et al. 1991). In brief, cells in a 12-well plate were washed with PBS, lysed in 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS, and 0.1 mg/ml of proteinase K, and transferred into a 1.5-ml Eppendorf tube. After 5 hr of incubation at 55°C with shaking, an equal volume of isopropanol was added, and DNA was allowed to precipitate by gentle inversion of the tube several times. The supernatant was discarded, and the DNA was washed with 70% ethanol, air-dried, and resuspended in distilled water. DNA (15 μg) was digested in 30 μl of a restriction enzyme mixture (1× restriction buffer, 100 mg/ml of bovine serum albumin (BSA), 50 μg/ml of RNaseA, and 15 units of *Hin*dIII) overnight at 37°C. Electrophoresis and Southern blotting of the digested DNA was performed as described previously (Sambrook et al. 1989). A ^{32}P-labeled 1.1-kb *Bam*HI fragment, which lies 3′ of the genomic sequence in the targeting vector, identified bands of 10.0 and 3.0 kb, or 10.0 and 4.6 kb, corresponding to germ-line wild-type *Brca1* and homologous recombinant bands. Targeted clones were recovered from the 24-well plates and expanded in 6-well plates. DNA from these clones was then digested with *Bam*HI and *Sal*I and probed with a 5′ flanking probe. In addition, a *neo* probe was used to confirm that only homologous recombination had occurred in targeted clones rather than a random integration event.

Generation of mice carrying the disrupted Brca1 *allele*

Two different targeted ES clones, Brca1–ko(o) 3 and Brca1–ko(s) 291, were used to generate chimeric mice according to procedures described previously (Bradley 1987). C57BL/6J blastocysts injected with 10–12 ES cells were implanted into pseudopregnant F_1 (CBA×C57BL/6) foster mothers (The Jackson Laboratory, Bar Harbor, ME). Chimeric mice, identified by agouti coat color, were mated with C57BL/6J mice. Offspring with agouti coat color were tested for the presence of the targeted locus by PCR and Southern blotting analysis. Heterozygotes were interbred, and PCR analysis was used to distinguish between offspring with zero, one, or two copies of the mutant gene.

DNA isolation, PCR genotyping, and histology of embryos from Brca1 *+/− intercrosses*

F_1 mice heterozygous for the *Brca1* mutant allele were mated, and toes were cut from the F_2 progeny for genotyping analysis. For timed pregnancies, the day on which a vaginal plug was detected was considered to be E0.5. At desired time points, the embryos were dissected from maternal decidua for further analysis. For embryos older than E8.5, the visceral yolk sac was collected and subjected to genotype determination by PCR. DNA from toes, yolk sacs, and blastocysts was prepared and analyzed by PCR as follows. Tissues were lysed at 55°C in 40 μl of lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml of gelatin, 0.45% NP-40, 0.45% Tween 20, and 60 μg/ml of proteinase K] for 1 hr. Samples were then boiled for 10 min and cooled on ice. Seven microliters of proteinase K-digested cell lysate was mixed with 18 μl of PCR cocktail solution containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 200 μM each of the four dNTPs, 0.4 μM of each primer, and 0.5 unit of recombinant *Taq* polymerase (AmpliTaq; Perkin-Elmer). The mixture was then overlaid with 20 μl of light mineral oil. PCR was performed for 35 cycles using a pTC-100 thermal controller (MJ Research, Inc.) using the following reaction conditions: Denaturing temperature of 94°C for 30 sec; annealing temperature of 65°C for 1 min; and elongation temperature of 72°C for 1 min. The resulting PCR products were resolved on 4% NuSieve/SeaKem (3:1; FMC) composite gel in TAE buffer at 80 V for 30 min and visualized with UV light after staining with ethidium bromide. For the targeted allele, a 236-bp product was generated using a sense oligonucleotide, 5′-TGATATTGCTGAAGAGCTTGGCGGC-3′ and an antisense oligonucleotide, 5′-TGGGAGTGGCACCTTCCAGGGTCAA-3′, within the *pgkneopA* cassette. To detect the wild-type allele,

a 150-bp product was generated using a sense oligonucleotide, 5′-AACAGCCTGGCATAGCAGTGAGCCA-3′, and antisense oligonucleotide, 5′-TTGCGGGTGAGTCCACTTCTCTCTA-3′ within exon 11 of *Brca1*. For embryos between E4.5 and E7.5, entire uteri were fixed in 4% paraformaldehyde overnight at 4°C. Uterine horns were excised and dehydrated through a graded ethanol series, cleared in chloroform, and then infiltrated and embedded in Paraplast X-tra (Polysciences). Sections (4 μm) were collected on Superfrost/Plus microscope slides (Fisher Scientific), stained with Mayer's hematoxylin/eosin, and mounted in Canada Balsam (Fisher Scientific). Images were recorded using a C5810 Color Camera (Hamamatsu) and cropped using a Macintosh Power PC and Adobe Photoshop Software. The slides with sections were demounted in xylene followed by soaking in 100% ethanol and air-dried. Embryonic tissues were microdissected and collected into glass capillaries (~50 μm diam.) mounted on a micromanipulator (Leitz), under 400-fold magnification, and transferred into a 0.5-ml Eppendorf tube containing 7 μl of lysis buffer under 20 μl of light mineral oil. Cells were lysed at 55°C for 1 hr. The proteinase K was then inactivated by incubation at 95°C for 10 min, and the mixture was then cooled on ice. The PCR cocktail solution (18 μl) was then added into the tube and mixed with the DNA sample by centrifugation at 12,000 rpm for 1 min. PCR was performed for 50 cycles.

In vitro blastocyst culture

Blastocyst cultures followed those described previously (Hsu 1979), with some modifications. Briefly, blastocysts were isolated from females at E3.5 and cultured for 5 days on tissue culture plates in DMEM plus 20% fetal bovine serum, supplemented with BSA (4 mg/ml), glutamine, antibiotics, and 2-mercaptoethanol (0.1 mM). Blastocyst outgrowths were inspected daily and photographed to monitor their development. Finally, they were lysed and genotyped by PCR.

Detection of BrdU incorporation by immunohistochemistry

At the desired time points, BrdU (100 μg/gram body weight) (Sigma) was injected intraperitoneally into pregnant females. One hour later, the entire uterus was dissected and fixed in 4% paraformaldehyde overnight at 4°C. The individual decidua were embedded, sectioned, and subjected to further analysis. Immunohistochemical detection of BrdU incorporation was performed as described previously (Lee et al. 1994). The percentage of cells incorporating BrdU in each embryo was determined by counting >50 cells in representative histological sections. Statistical comparisons were performed by two-tailed ANOVA using In Stat software (Graph Pad, San Diego, CA)

Acknowledgments

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The Nuclear Localization Sequences of the BRCA1 Protein Interact with the Importin-α Subunit of the Nuclear Transport Signal Receptor*

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Summary

The *BRCA1* gene product is a nuclear phosphoprotein that is aberrantly localized in the cytoplasm of most breast cancer cells. In an attempt to elucidate the potential mechanism for the nuclear transport of BRCA1 protein, three regions of highly charged, basic residues, ^{503}KRKRRP^{508}, ^{606}PKKNRLRRKS^{615} and ^{651}KKKKYN^{656} were identified as potential nuclear localization signals [NLS]. These three regions were subsequently mutated to ^{503}KLP^{508}, ^{607}KLS^{615}, and ^{651}KLN^{656}, respectively. Wild-type and mutated proteins were tagged with the flag-epitope, expressed in human DU145 cells and detected with the M2 monoclonal antibody. In DU145 cells, the KLP mutant completely fails to localize in nuclei, whereas the KLS mutant is mostly cytoplasmic with occasional nuclear localization. The KLN protein is always located in nuclei. Consistently, hSRP1α (importin-α), a component of the NLS receptor complex, was identified in a yeast two-hybrid screen using BRCA1 as the bait. The specificity of the interaction between BRCA1 and importin-α was further demonstrated by showing that the ^{503}KRKRRP^{508} and ^{606}PKKNRLRRKS^{615} regions, but not ^{651}KKKKYN^{656}, are critical for this interaction. To determine if the cytoplasmic mislocation of endogenous BRCA1 in breast cancer cells is due to a deficiency of the cells, wild-type BRCA1 protein tagged with the flag-epitope was ectopically expressed in six breast cancer cell lines. The analysis demonstrated that, in all six, this protein localized in the cytoplasm of these

cells. In contrast, expression of the construct in four non-breast cancer cell lines resulted in nuclear localization. These data support the possibility that the mislocation of the BRCA1 protein in breast cancer cells may be due to a defect in the cellular machinery involved in the NLS-receptor-mediated pathway of nuclear import.

Introduction

BRCA1, located on chromosome 17q21, was cloned and shown to be responsible for about 50% of familial breast and ovarian cancers (1). The protein encoded by this gene contains a zinc finger motif and an acidic block of residues (1). These features suggest that BRCA1 may function as a transcription factor, although there is no experimental evidence so far to support this supposition. Similarly, the precise biological function of BRCA1 protein remains unclear. There are several lines of circumstantial evidence, however, suggesting that BRCA1 may have a role in cellular growth and differentiation. First, BRCA1 mRNA is highly expressed in tissues where cells are rapidly proliferating and differentiating (2,3). Second, homozygous deletion of the *BRCA1* gene in mice causes lethality in early embryogenesis due to the retardation of cell growth and malformation of the embryo (4-6). Third, the expression of both BRCA1 mRNA and protein is increased following cell cycle progression. Moreover, phosphorylation of the BRCA1 protein by cyclin-dependent kinases is also positively regulated during the cell cycle (7).

As a tumor suppressor gene, it is unusual that mutations in *BRCA1* are clearly linked to inherited breast and ovarian cancers, but are rarely found in sporadic tumors (1,8,9). This result has raised questions concerning the authenticity of the *BRCA1* gene as a

breast tumor suppressor (10). However, inactivation of tumor suppressor proteins can be independent of their genetic mutations. For example, wild-type p53 protein has also been found mislocated in the cytoplasm of breast cancer cells, while mutant p53 remains in the nucleus (11). These studies suggested the existence of multiple pathways for the inactivation of p53 function in breast cancer cells. It is therefore possible that BRCA1 may be functionally inactivated by its mislocation from the nuclear to cytoplasmic compartment in sporadic breast cancer cells.

Nuclear transport is a complicated process involving multiple factors. Being a large molecule, it is likely that the BRCA1 protein is actively translocated from the cytoplasm to the nucleus by the NLS-receptor-mediated-transport system [Reviewed in (12,13)]. The direct import of karyophilic proteins through the nuclear pore complex requires energy (14,15) and an NLS located in the transport substrate (16,17) to which a cytosolic receptor complex, importin-α and importin-β, binds (18,19). A GTP-binding protein, RAN, mediates the energy-dependent translocation of the substrate-receptor complex through the nuclear pore complex (20). After translocation, importin-β dissociates from the complex in the vicinity of the inner aspect of the nuclear envelope while importin-α accompanies the substrate to its sites of function (21). Any defect in this transportation system could lead to a failure in the translocation of BRCA1 to the

nucleus. However, it is very unlikely that the cells could survive a major deficit in nuclear transport.

In contradiction to the findings of ourselves (22,23) and others (24), it has been reported that BRCA1 is a secreted protein (25). Since the subcellular location of proteins is a fundamental aspect of their function, it is important to solidify the data regarding the location of BRCA1 in normal and cancer cells. In an attempt to address these questions, we initiated experiments to investigate the nuclear transport of the BRCA1 protein by ascertaining the identity of its functional NLS motifs, by identifying proteins with which it interacts, and by extending the investigation of its subcellular distribution in breast cancer cells. Our results indicate that there are two functional nuclear localization sequences in the BRCA1 protein that interact with hSRP1α [importin-α or karyopherin-α (18,26,27)]. Furthermore, ectopically expressed wild-type BRCA1 protein is located in nuclei of normal and non-breast tumor cell lines, but is detected in the cytoplasm of all breast cancer cell lines tested.

Experimental Procedures

Cell culture and DNA transfections. Human cell lines DU145 [prostate cancer], T24 [bladder cancer], T47D, ZR75, MB231, MB468, MDA330, MCF7 [breast cancer], HBL100 [normal breast epithelial cells immortilized with SV40] and CV1 [monkey kidney cell line] were grown at 37°C in a humidified 10% CO_2-containing atmosphere in Dulbecco's modified Eagle's medium [DMEM, Gibco] supplemented with 10% heat inactivated fetal calf serum [Flow Laboratories] on plastic surfaces. Each 10 cm dish of cells grown to 60% confluency was transfected with 10 μg of plasmid DNA using the calcium phosphate method (28). The calcium phosphate precipitate was left in the culture medium for six to eight hours. At that time the medium was drained and the cells were refed with fresh medium.

NLS Mutagenesis. To introduce mutations into the three putative nuclear localization sequences of BRCA1, a PCR-based strategy was used. Briefly, the following external and internal primers with *Hin*dIII restriction sites [underlined, below] were used to create in-frame deletions of each NLS sequence, replacing the deletion with a single leucine residue. The external primers used for all of the NLS mutations were 5'-GATTTGAACACCACTGAGAAGCGTGCA [733 to 759 of BRCA1 cDNA] and 5'-CTTTAAGGACCCAGAGTGGGCAGAGAA [2679 to 2653]. For the KLP mutation the

following internal primers were used. 1A: 5'-CCTTTTAAGCTTTAATTTATTTGTGAGGGGACGCTC [1506 to 1483] and 1B 5'-CCTTAAAGCTTCCTACATCAGGCCTTCATCCTGA [1522 to 1544]. For the KLS mutation the following internal primers were used. 2A 5'-CCTCCCAAGCTTAGGTGCTTTTGAATTGTGGATATTT [1818-1794] and 2B 5'-CCTCCCAAGCTTTCTTCTACCAGGCATATTCATGCGC [1843 to 1867]. The KLN mutation was generated with the following internal primers. 3A 5'-CCTCCCAAGCTTTATCTCTTCACTGCTAGAACAACT [1950 to 1927] and 3B 5'-CCTCCCAAGCTTAACCAAATGCCAGTCAGGCACAGC [1966 to 1989]. Plasmid BSK-BRCA1a that contains a full-length BRCA1 cDNA (7) was used as the template for PCR amplifications using each pair of internal and external primers. The resulting DNA fragments were gel purified and cut with *Afl*II and *Hin*dIII for the N-terminal cDNA fragments, and with *Kpn*I and *Hin*dIII for the C-terminal cDNA fragments. The *Afl*II and *Hin*dIII restriction sites are within the BRCA1 DNA sequence downstream and upstream of each 5' and 3' external primers, respectively. The N- and C-terminal fragments were then used to replace the AflII/KpnI fragment in pBSK-BRCA1a. Ligation of the *Hin*dIII site at each of the NLS sites generated in-frame deletions, with the addition of a CTT codon for leucine. The *Afl*II/*Kpn*I fragments from pBSK-BRCA1-KLP, KLS, and KLN were then used to replace a similar fragment in the expression

vector pCEP-flag-BRCA1 (23) to generate pCEP-flag-$BRCA1_{KLP}$, pCEP-flag-$BRCA1_{KLS}$ and pCEP-flag-$BRCA1_{KLN}$.

Transient Expression and Immunostaining. Cells were transfected with pCEP-flag-$BRCA1_{KLP}$, pCEP-flag-$BRCA1_{KLS}$ or pCEP-flag-$BRCA1_{KLN}$ for expression of the flag-tagged NLS mutated proteins and pCEP-flag-BRCA1 for flag-tagged wild-type BRCA1. Thirty hours after transfection, the cells were fixed and indirectly immunostained with the anti-flag M2 mAb [Kodak, Rochester, N.Y.] using previously described procedures (29). The microscopic images were acquired using a Hammamatsu Color Chilled 3CCD camera attached to a Zeiss Axiophot fluorescence microscope. The image files were digitally processed for presentation using Adobe Photoshop.

Western Blots. Thirty hours after transfection, the cells were lysed with 250 lysis buffer (7) and denatured by boiling in sample buffer for five minutes. After SDS/PAGE, flag-tagged BRCA1 protein was detected in Western blots using the anti-flag M2 mAb and endogenous BRCA1 was detected with the monoclonal antibody 6B4 (23).

Identification of an activation domain within BRCA1. The identification of an activation domain in BRCA1 was done by a yeast one-hybrid assay in *S. cerevisiae* strain Y153, which contains a *lacZ* reporter under the control of a promoter with GAL4-

binding sites in the upstream activating sequence of GAL1 [UAS_G] (30). The BRCA1 deletion constructs in Figure 2 were obtained by translationally fusing the DNA-binding domain of GAL4 (31,32) in pAS (30) to cDNA fragments obtained from pBSK-BRCA1a (7) using convenient restrictions sites. β-galactosidase activity was determined by colony color and quantitated using chlorophenyl-red-b-D-galactopyranoside [CPRG] in assays as previously described (30).

Yeast two-hybrid screen. A cDNA library prepared from human B lymphocytes was screened as previously described (30). The protein from pAS-BRCA3.5 [Figure 2] served as the "bait" which consisted of amino acids 1 to 1142 of BRCA1 fused to the GAL4 DNA-binding domain (31,32) in plasmid pAS (30).

Interactions between the NLS of BRCA1 and importin-α. Yeast strain Y153 was co-transfected with pAS-BRCA3.5, pAS-KLP, pAS-KLS or pAS-KLN and pACT-$importin_{220-529}$ [Figure 3A] and assayed for β-galactosidase activity as described (30). For importin-α expression, a cDNA encoding amino acids 220 to 529 was fused to the activation domain of GAL4 (31,32) in pACT (30). pAS-KLP, pAS-KLS and pAS-KLN were constructed by fusing $BRCA1_{1-1142}$ cDNAs from pBSK-BRCA1-KLP, KLS, and KLN to the DNA-binding domain of GAL4 in pAS (30). β-galactosidase activity was assayed as described above.

Results

Determination of a nuclear Localization Sequence in BRCA1. To initiate the study of BRCA1 nuclear transport, we began with the identification of its NLS motif[s]. By analysis of the amino acid sequence, three possible nuclear localization sequences with highly charged, basic residues were found in BRCA1, 503-KRKRRP-508, 606-PKKNRLRRKS-615 and 651-KKKKYN-656 [Figure 1A]. To determine if these sequences are functional in nuclear localization, PCR-based mutagenesis [See Methods] was performed that generated in-frame deletions, and replacement with a single leucine residue at each of the sites [Figure 1A]. The wild-type and mutated BRCA1 proteins were tagged by fusion with the flag epitope in a pCEP based plasmid. The expression of these tagged proteins in human DU145 cells was done by transient transfection, and the proteins were detected by immunoblotting with either anti-BRCA1 mAb 6B4 [Figure 1B, lanes 1-5] or anti-flag M2 mAb [Figure 1B-lanes 6-10]. As shown in Figure 1-B, anti-flag M2 mAb detected only ectopically expressed flag-tagged BRCA1, which co-migrates with endogenous BRCA1 as a 220 kDa protein [lanes 1-5] in each population of transfected cells [lanes 7-10] but not in untransfected cells [lane 6]. This result indicated that all the plasmid constructs transfected into DU145 cells were capable of expressing flag-tagged BRCA1 proteins, which were either wild-type or mutated.

The subcellular localization of wild-type and each of the mutated proteins was determined by immunostaining with the anti-flag M2 mAb. Consistent with its previous localization (22), wild-type flag-tagged BRCA1 protein is located in the nucleus [Figure 1C-a and b]. The 651-KLN-656 mutant [Figure 1C-g and h] is also nuclear, indicating that the residues 651-KKKKYN-656 are not important for nuclear transport of the flag-$BRCA1_{KLN}$ protein. In contrast, the 503-KLP-508 and 607-KLS-615 mutations both resulted in cytoplasmic localization of flag-$BRCA1_{KLP}$ and flag-$BRCA1_{KLS}$ proteins [Figure 1C-c and d, -e and f, respectively], indicating that both of these stretches of basic residues are critical for nuclear import. It was noted that flag-$BRCA1_{KLS}$, when overexpressed, can, in some instances, localize in the nucleus. This is illustrated in Figure 1C, panels e and f, where two highly expressing cells are adjacent to each other, one shows cytoplasmic staining and the other nuclear [arrow heads]. However, flag-tagged $BRCA1_{KLP}$ was never observed in the nucleus.

Identification of BRCA1-Interacting Proteins. Nuclear transport of BRCA1 clearly requires interactions with other cellular proteins. We elected to use the yeast two-hybrid method to identify and clone genes encoding BRCA1-interacting proteins. Since BRCA1 has been proposed to be a transcription factor (1) it may therefore have transactivation activity. The presence of such activity would confound a two-hybrid assay. To functionally identify potential transactivation domains in BRCA1, various

domains of BRCA1 protein were fused in-frame with the DNA-binding domain of GAL4 [Figure 2] in plasmid pAS (30). If these fusion proteins contain an activation domain, they will activate the GAL4 UAS_G-responsive β-galactosidase reporter (30) after transfection into the Y153 strain. Through this analysis we defined a strong activation domain located between amino acids 1142 to 1646, Figure 2. This activation domain was deleted in BRCA3.5 [Figure 2] which only contains amino acid 1 to 1142 of BRCA1. BRCA3.5 was then fused to the GAL4 DNA-binding domain of pAS vector as the bait for screening BRCA1-interacting proteins as previously described (30). Four different clones were isolated and sequenced. When compared to currently available GenBank, we found that one is novel, one has homology to an uncharacterized zinc-finger domain-containing protein, and two bear sequence homology to previously cloned cDNAs [Table 1]. Interestingly, the sequence of hBRAP21 is identical to that of the nuclear localization signal receptor hSRP1α (26), also known as importin-α (18) or karyopherin-α (27).

Interaction of importin-a with BRCA1. To investigate the potential interaction of BRCA1 with importin-α and to further confirm the functional NLS of BRCA1, we tested the specificity of the interaction of BRCA1 and importin-α using the yeast two-hybrid method. To do this, either wild-type BRCA3.5 from amino acid 1 to 1142, or the same region containing the mutated NLS sequences [KLP, KLS and KLN, See Figure 1A] was

translationally fused to the DNA-binding domain of GAL4 in the pAS expression vector [Figure 3A]. In addition, the region of importin-α from amino acid 220 to 529, which is known to interact with BRCA3.5, was fused to the activation domain of GAL4 in pACT [Figure 3A] (30). The various BRCA1 proteins were co-expressed with the importin-α in the Y153 cells. A strong interaction between importin-α with BRCA3.5 or the KLN mutant was observed by the generation of blue colonies and an increase in β-galactosidase activity about 100 fold over that of the negative control, untransfected Y153 cells [Figure 3-B]. These results are consistent with the observation that flag-$BRCA1_{KLN}$ is localized in nuclei [Figure 1C, panels g and h]. However, the KLP mutant failed to interact with importin-α and resulted in white colonies and no increase of β-galactosidase activity over background, Figure 3-B. Interestingly, a ten-fold increase in β-galactosidase activity over background was observed with the KLS mutant [Figure 3-B]. As noted earlier, this increase in activity is consistent with the immunostaining data for the BRCA1 protein containing this mutation, which showed occasional nuclear localization when overexpressed [Figure 1C, panels e and f].

Cytoplasmic Localization of Ectopically Expressed BRCA1 in Breast Cancer Cells. Previously, we transfected an expression plasmid containing flag-tagged BRCA1 into two breast cancer cell lines, T47D and MB468, and one immortalized non-breast epithelial cell line, HBL100. The flag-tagged BRCA1 protein was found in the cytoplasm

of the T47D and MB468 cells and the nucleus of HBL100 cells by immunostaining with anti-flag M2 monoclonal antibody. To confirm this observation and to verify the expression of full-length flag-tagged BRCA1 protein, we repeated this experiment using four non-breast cancer and six breast cancer cell lines listed in Table 2. As shown in Figure 4 [summarized in Table 2], nuclear localization of flag-BRCA1 is observed in normal monkey kidney cells CV1, [Figure 4-a-b] and in DU145, T24, and HBL100 cells [Table 2]. In contrast, cytoplasmic localization of this protein is seen in ZR75 and MB231 [Figure 4, panels c, d and e, f, respectively], and in MB468, MDA330 and MCF7 breast tumor cells [Table 2]. These data suggest an altered transport or retention system for the BRCA1 protein in breast cancer cells.

Discussion

BRCA1 is a nuclear protein. The identification of two regions of charged, basic amino acids between 503 to 508, and 606 to 615 that are both crucial for efficient nuclear transport of the BRCA1 protein further supports this notion. The distance between these two motifs is much greater than the ten amino acids separating the bipartite sites of nucleoplasmin (33). The structure and function of the NLSs in BRCA1 is similar to other nuclear proteins in which two NLSs are more widely spaced such as those in the polyoma large T antigen (34), influenza A virus NS1 protein (35) and adenovirus DNA-binding protein (36). While we cannot rule out the possibility that other sequences are also required for translocation of BRCA1 from the cytoplasm to the nucleus, the NLS at 503-508 is essential for this process. This observation was supported by results showing that mutation of the NLS at 503-508 in BRCA1 completely abolished its interactions with importin-α. The NLS at 606-615 in BRCA1 is less critical because mutation of this NLS did not completely diminish the nuclear import of BRCA1. Our results that BRCA1 is a nuclear protein with a functional NLS are at odds with the report indicating that the protein is membrane-bound, and secreted (25). Such a discrepancy is puzzling but may be explained by cross-reactivity of the peptide antisera to the EGF receptor (37).

Using mouse polyclonal antibodies specific for the BRCA1 protein we have consistently found BRCA1 to be a 220 kDa nuclear protein that is aberrantly located in the cytoplasm of advanced breast cancer cells (22,23). However, Scully, et al., (24) reported that the 220 kDa BRCA1 protein remains in the nucleus of some breast cancer cell lines. Although the precise reason for this discrepancy is unclear, one cannot exclude the possibility of less specific antibodies, potential immunostaining artifacts, or both. By ectopically expressing epitope-tagged BRCA1 protein and using the specific anti-flag M2 monoclonal antibody we have circumvented the difficulties in obtaining highly-specific antibodies against BRCA1. Through this completely different approach, wild-type flag-tagged BRCA1 expressed in breast cancer cells remains in the cytoplasm. This result further suggests that its mislocation in breast cancer cells is not due to mutations of *BRCA1* itself. Rather the aberrant localization seems to be the result of alterations in the cells, perhaps at the level of nuclear transport of BRCA1.

In this regard, the demonstration here that BRCA1 interacts with the importin-α subunit of the nuclear transport receptor complex could be an important clue. However, if there is a problem with the importin-α subunit or the importin-substrate complex, why is it manifested in breast epithelial cells? Does this indicate an unsuspected specificity of importin-α for BRCA1? And, does the defect in the function of BRCA1 reside in the cytoplasm or nucleus? Once translocation across the nuclear pore complex occurs, importin-α is reported to accompany the transport substrate to its areas of nuclear

function (21). If there is a problem with BRCA1 dissociating from importin-α in the nucleus, perhaps BRCA1 protein is immediately exported from the nucleus, resulting in the appearance of cytoplasmic localization. Co-localization experiments similar to those in Gorlich, et al., (21) using normal and breast cancer cells might address this possibility.

An alternative possibility is that, in breast cancer cells, there is a problem in the regulation of the nuclear transport of BRCA1. The known mechanisms for regulating nuclear translocation [Reviewed in (12,13)] are: [a] phosphorylation/dephosphorylation, e.g., *c-rel* and *v-jun* and cell-cycle regulated proteins such as cyclin B/Cdk complex and Pendulin; [b] cytoplasmic retention by masking of the NLSs as seen in *dorsal*, NFkB, the glucocorticoid receptor and the *periodicity* protein; or [c] more general regulation at the level of the nuclear pore complex. Perturbations in the gene products in any of these regulatory systems could potentially result in cytoplasmic localization of BRCA1 in breast epithelial cells. The possibility that some of the other BRCA1-interacting proteins identified in the two-hybrid screen could have this kind of role in breast cancer cells is being investigated.

Whatever the pathogenic alteration in breast cancer cells is, it is not, at this time, obvious whether the mislocation of BRCA1 is the cause of or the result of the tumor phenotype. Interestingly, there are other reports of mislocation to the cytoplasm of a nuclear tumor suppressor protein in breast and other types of cancer cells. Of 27 breast cancer cases examined, 37% demonstrated cytoplasmic staining for p53, which by

sequencing was revealed to be wild-type (11). In another study, wild-type p53 was found located in the cytoplasm of human cervical carcinoma cell lines with integrated human papillomavirus-18 or 16 (38). Both of these studies suggest that the tumor suppressor function of normal p53 can, in some cases, be inactivated by cytoplasmic mislocation (11,38). These data are similar to our observations for BRCA1 and seem to suggest a global alteration of subcellular compartmentation in breast cancer cells. If this is the case, then BRCA1 and p53 along with, perhaps, other nuclear regulatory proteins may be retained in the cytoplasm of these cells, the composite effect of which may contribute to their tumorigenesis.

FIGURE LEGENDS

Figure 1. *Nuclear localization sequence in BRCA1.* A. Schematic of the epitope [flag] -tagged BRCA1 protein showing the approximate location and sequence of the three putative NLS sequence motifs. Also shown are the sequence and coordinates of the mutations that were introduced into the BRCA1 protein by PCR mutagenesis [described in Materials and Methods]. B. Expression of full-length epitope-tagged wild-type and mutated BRCA1 proteins. BRCA1 proteins were detected in immunoblots developed with the anti-BRCA1 6B4 mAb [Lanes 1-5] or the anti-flag M2 mAb [Lanes 6-10]. The arrow shows the position of the 220 kDa endogenous and exogenous BRCA1 proteins. C. DU145 cells transfected with pCEP-flag-BRCA1 [a, b], pCEP-flag-BRCA1$_{KLP}$ [c, d] pCEP-flag-BRCA1$_{KLS}$ [e, f] and pCEP-flag-BRCA1$_{KLN}$ [g, h], were processed for immunostaining with the anti-flag M2 mAb [panels b, d, f and h]. Panels a, c, e and g show DAPI staining of the same cells to indicate the location of the nucleus. As shown in panel f, high expression of the flag-tagged BRCA1$_{KLS}$ protein can, in a small number of cases, result in nuclear staining of the DU145 cells [arrow heads]. The microscopic images were acquired as described in Material and Methods.

Figure 2. *Identification of transactivation domain of the BRCA1 protein.* The top diagram illustrates the BRCA1 protein and indicates the position of the zinc finger,

putative NLSs and the acidic domain. Also shown are the truncated BRCA1 cDNAs [indicated by coordinates on each line] translationally fused to the DNA-binding domain of GAL4 (31,32) [hatched open boxes] in the pAS plasmid (30). Each plasmid [indicated to the left of each construct] was expressed in the Y153 strain of yeast (30). Shown are the colony colors and β-galactosidase activities which were measured as described in Materials and Methods.

Figure 3. *Importin-α interactions with wild-type and mutant BRCA1 with nuclear localization sequence deletions.* **A**. Diagrams of the constructs used in the yeast two-hybrid assays. pAS-BRCA3.5 was described previously [Figure 2]. pAS-KLP, KLS, and KLN are shown with the coordinates for each of the mutations in BRCA1 which was fused to the DNA-binding domain [DBD] of GAL4 in pAS (30). Also illustrated is pACT-importin containing the 229 to 529 amino acid region of importin-α [stippled rectangle] fused to the transactivation domain of GAL4, TAD (31,32) [solid hatched box]. **B**. Results of the assays including colony colors and levels of β-galactosidase activity. Y153 denotes the negative control cells that were not transfected.

Figure 4. *BRCA1 is located in the cytoplasm of breast cancer cells.* Immunostaining [panels b, d and f] of the flag-tagged BRCA1 protein in normal monkey kidney cells, CV1, [panels a and b], breast cancer cell lines ZR75 [panels c and d] and MB231 [panels

e and f] cells are illustrated. DAPI staining is shown in panels a, c and e to indicate nuclei of the cells.

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An alternative splicing event, which occurs in mouse pachytene spermatocytes, generates a form of DNA ligase III with distinct biochemical properties that may function in meiotic recombination.

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Running title: **Testis-specific form of DNA ligase III**

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Abstract

Three mammalian genes encoding DNA ligases have been identified. However, the roles of each of these enzymes in mammalian DNA metabolism has not been established. In this study we show that two forms of mammalian DNA ligase III, α and β, are produced by a conserved tissue-specific alternative splicing mechanism involving exons encoding the C-termini of the polypeptides. DNA ligase III-α cDNA, which encodes a 103 kDa polypeptide, is expressed in all tissues and cells whereas DNA ligase III-β cDNA, which encodes a 96 kDa polypeptide, is only expressed in the testis. During male germ cell differentiation, elevated expression of DNA ligase III-β mRNA is restricted, beginning only in the latter stages of meiotic prophase and ending in the round spermatid stage. In 96 kDa DNA ligase III-β, the C-terminal 77 amino acids of DNA ligase III-α are replaced by a different 17-18 amino acid sequence. As reported previously, the 103 kDa DNA ligase III-α interacts with the DNA strand-break repair protein encoded by the human *XRCC1* gene. In contrast, the 96 kDa DNA ligase III-β does not interact with XRCC1 indicating that DNA ligase III-β may play a role in cellular functions distinct from the DNA repair pathways involving the DNA ligase III-α·XRCC1 complex. The distinct biochemical properties of DNA ligase III-β, in combination with the tissue and cell-type specific expression of DNA ligase III-β mRNA, suggest that this form of DNA ligase III is specifically involved in the completion of homologous recombination events that occur during meiotic prophase.

Introduction

The joining of DNA single-strand breaks is an essential step in the completion of lagging strand DNA synthesis and DNA excision repair pathways. Additionally, exchanges between homologous DNA duplexes, which are completed by the cleavage of Holliday junctions, require DNA joining events to generate intact recombinant molecules.

Three human genes encoding DNA ligases have been identified (3, 9, 42). Genetic and biochemical studies on the product of the *LIG1* gene indicate that this enzyme functions to join Okazaki fragments during DNA replication (3, 4, 28, 34, 39, 43). The sensitivity of the DNA ligase I-mutant cell line 46BR to DNA damage by alkylating agents and the abnormal repair of uracil-containing DNA substrates by 46BR cell-free extracts implicate DNA I ligase in DNA base excision repair (4, 18, 22, 28, 35). The recent characterization of an interaction between DNA polymerase β, which is essential for base excision repair of alkylation damage in mammalian cells (33), and DNA ligase I within a multiprotein complex that catalyzes the repair of a uracil-containing DNA substrate provides evidence at the molecular level that DNA ligase I is involved in DNA base excision repair (27).

The *LIG3* and *LIG4* genes encode polypeptides that have similar electrophoretic mobilities in denaturing polyacrylamide gels (42). These gene products with molecular masses of about 100 kDa can be distinguished by the ability of DNA ligase III to form a stable complex with the product of the human *XRCC1* gene (7, 8, 42). Human *XRCC1* was cloned by its ability to complement the hypersensitivity of the Chinese hamster ovary cell line, EM9, to DNA alkylating agents (36, 37). Because the EM9 cell line is defective in the joining of DNA single-strand breaks and contains reduced levels of DNA ligase III activity, it appears that DNA ligase III functions in the repair of DNA single-strand breaks that arise either by the direct action of a DNA damaging agent, such as ionizing radiation, or as a consequence of DNA repair enzymes excising lesions (7, 8, 23, 36, 37). At the present time, there is very little known about the cellular role of DNA ligase IV.

Analysis of the steady state levels of DNA ligases I and III mRNAs in different human tissues and cells revealed that both of these genes are highly expressed in the testis. In

developing mouse testis, the high levels of DNA ligase I expression correlate with the contribution of proliferating spermatogonia to the testis and suggest that, as in proliferating somatic cells, DNA ligase I functions in DNA replication (9). In contrast, the high levels of DNA ligase III expression correlate with the appearance and accumulation of cells undergoing meiotic recombination. This association between DNA ligase III expression and meiotic cells is supported by the specific labeling of primary spermatocytes in mouse testis sections by in situ hybridization with a DNA ligase III antisense probe (9). Furthermore, expression of the *XRCC1* gene, whose product interacts with DNA ligase III (7, 8, 42), is also elevated in both pachytene spermatocytes and round spermatids (40, 41).

It is possible that DNA ligase III is specifically involved in the completion of meiotic recombination events. Alternatively, or in addition, DNA ligase III may repair DNA single-strand breaks which are introduced as a consequence of either changes in chromatin structure or DNA damage that occur during the latter stages of germ cell development. In this report, we demonstrate that a testis-specific alternative splicing mechanism results in the synthesis of a DNA ligase III polypeptide with distinct biochemical properties. We suggest that one form of DNA ligase III functions in a complex with XRCC1 to repair DNA single-strand breaks in all tissues and cells, whereas the testis-specific form is involved in the completion of meiotic recombination events in male germ cells.

Materials and Methods

Cloning of human and mouse DNA ligase III cDNAs- Human DNA ligase III cDNAs have been isolated from HeLa and testis cDNA libraries (9, 42). These cDNAs have different 3' ends and encode different-sized polypeptides with distinct C-termini (Fig. 1). Within the identical regions of the HeLa and testis DNA ligase III cDNAs, there is an internal EcoRI site at nucleotides 2452-2457 in the testis cDNA (9). The nucleotide sequences of the HeLa and testis cDNAs diverge 435 nucleotides 3' of the EcoR I site. Using the 600 bp sequence at the 3' end of the testis cDNA as a probe (EcoRI-XbaI fragment) (9), a 1.1 kb cDNA fragment was isolated from a human liver cDNA library (Stratagene). The DNA sequences of both strands of

this cDNA fragment were determined and found to be identical to the 3' end of the HeLa DNA ligase III cDNA (42). A cDNA that encodes the same 922 amino acid polypeptide as the HeLa DNA ligase III cDNA was constructed by replacing the 3' 600 bp EcoRI-XbaI fragment of the testis DNA ligase III cDNA with the 1.1 kb EcoRI fragment from the liver cDNA library. This form of DNA ligase III has been designated DNA ligase III-α.

Mouse DNA ligase III cDNAs have been isolated from a mouse testis cDNA library (Clontech) using human DNA ligase III cDNA as a probe (9). A full-length cDNA (3080 bp), which contains an internal EcoRI site at the same position as the one in the human DNA ligase III cDNAs, encodes a polypeptide that is similar in size (868 residues, calculated molecular weight of 96,000) to and homologous with the polypeptide encoded by human testis DNA ligase III cDNA (9). This form of DNA ligase III has been designated DNA ligase III-β. The mouse DNA ligase III-β cDNA sequence has been deposited in the GenBank (accession no. U66057).

Using oligonucleotides GATGAGACGCTGTGCCAAA and GGAAGACAGCAAACCTAGC that correspond to nucleotides 2863-2881 and the complement of nucleotides 3134-3016 of human DNA ligase III-α cDNA, respectively (Fig. 1A) and the 1.1 kb EcoRI fragment from the human liver cDNA library as a template, a 251 bp fragment was amplified by the polymerase chain reaction (PCR) (31). The PCR mixture (100 μl) contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each of the dNTPs, 1 μM of each of the oligonucleotides, 10 ng cDNA template and 2.5 units of Taq DNA polymerase (Boehringer Mannheim). PCR amplification was carried out by 30 cycles of 94°C for 1.5 min, 56°C for 2 min and 72°C for 2 min. Using the 251 bp DNA fragment as a probe, a 1.3 kb cDNA was isolated from the mouse testis cDNA library. The DNA sequence at one end of this cDNA is identical to that 3' of the EcoRI site in the mouse DNA ligase III-β cDNA described above for 438 nucleotides but then the sequences diverge. The nucleotide and predicted amino acid sequences after the point of divergence are homologous with the 3' end and predicted amino acid sequence of human DNA ligase III-α cDNA (Fig. 1A) (42)). A mouse DNA ligase III-α cDNA (3832 bp), which was constructed by replacing the 3' 600 bp of

sequence from the EcoRI site with the 1.3 kb fragment, encodes a polypeptide consisting of 927 amino acids that has a calculated molecular weight of 103,000. The mouse DNA ligase III-α DNA sequence has been deposited in the GenBank (accession no. U66058).

DNA sequencing and sequence analysis- Double-strand DNA sequencing was carried out by the dideoxy chain termination method with Sequenase and synthetic sequencing primers. The DNA sequences of both strands were determined with primers at intervals of about 150 bases. DNA sequences were aligned using Seqman and translated using Editseq (DNAStar). Nucleotide and amino acid sequence homologies were aligned with Align (DNAStar).

Cloning and analysis of mouse DNA ligase III gene- A 0.9 kb PstI fragment (nucleotides 1448-2333) from mouse DNA ligase III cDNA was used as a probe to screen a mouse 129/J genomic library (Stratagene). After gel purification, the 0.9 kb fragment was labeled (10^8 cpm/μg) with [α^{32}P]dCTP (Amersham) using the Prime-It II Random Primer Labeling Kit (Stratagene) according to the manufacturer's protocol. A total of 3 x 10^6 phage plaques were transferred to nitrocellulose filters and immobilized by UV-cross-linking prior to hybridization with the denatured probe (10^6 cpm/ml) in a solution containing 5X Denhardts solution, 6X SSC, 100 μg /ml salmon sperm DNA and 0.25% SDS for 16 h at 68°C (32). After hybridization, the filters were washed for 15 min once at room temperature and twice at 68°C in 1X SSC and 0.5% SDS. Additional rounds of screening were carried out to isolate a homogeneous phage population.

DNA was isolated from the single phage that hybridized to the 0.9 kb cDNA probe. This phage contained an 18 kb genomic fragment that was further analyzed by restriction enzyme mapping and Southern blotting (32) with probes from different regions of the DNA ligase III cDNA. The genomic fragment was found to contain exons encoding the C-terminal half of DNA ligase III.

Probes specific for the different 3' ends of mouse DNA ligase III cDNA were generated by the PCR. Using oligonucleotides ACTGTTGGATGTCTTCACTGGG and AAAGACAAAGCTAGCACCCGGA that correspond to nucleotides 2914-2935 and the complement of nucleotides 3349-3326 of the 3832 bp mouse DNA ligase III-α cDNA,

respectively (Fig. 1B) and the 3832 bp mouse DNA ligase III-α cDNA as a template, a 425 bp fragment was amplified by the PCR. The PCR conditions were as described above except the annealing step was carried out at 62°C. Using oligonucleotides CAGCCGGCAAAGAGGAAGGA and TTTTTGCTGCACCCCACCGC that correspond to nucleotides 2923-2942 and the complement of nucleotides 3073-3052 of the 3080 bp mouse DNA ligase III-β cDNA, respectively (Fig. 1B) and the 3080 bp mouse DNA ligase III-β cDNA as a template, a 150 bp fragment was amplified by the polymerase chain reaction (PCR). The PCR mixture (100 µl) contained 10 mM Tris-HCl (pH 8.9), 5 mM $MgSO_4$, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.2 mM of each of the dNTPs, 0.2 mM of each of the oligonucleotides, 40 ng cDNA template and 2.5 units of Pwo DNA polymerase (Boehringer Mannheim). PCR amplification was carried out by 30 cycles of 94°C for 1.5 min, 64°C for 2 min and 75°C for 2 min.

Enrichment of specific spermatogenic cell types- A standard StaPut gradient separation was employed to obtain enriched populations of late pachytene spermatocytes, round spermatids and residual bodies from the testes of adult male mice (29). Enriched populations of Sertoli cells and spermatogonia (types A and B) were isolated from testes of 8-day old mice while enriched populations of pre-leptotene, leptotene plus zygotene and early pachytene spermatocytes were isolated from 17- to 18-day old mice using a modified StaPut gradient system (5). Germ cell preparations from adult male mice were >90% homogeneous while preparations from younger animals were >85% homogeneous as determined by phase-contrast microscopy.

RNA Isolation- Poly (A^+) RNA was isolated from the testes of 5-, 8-, 15-, 25- and >60-day old mice and from purified germ cell populations as described previously (40). Total RNA was isolated from mouse tissues and purified germ cell populations by cesium chloride centrifugation after lysis in guanidinium isothiocyanate (12). RNA was quantitated by measuring absorbance at 260 nm.

Northern blot analysis- Poly (A^+) RNA (2.0 µg) was electrophoresed through a 1.2% agarose-formaldehyde gel, transferred to a nitrocellulose membrane and then immobilized on the membrane by UV cross-linking. The membrane was incubated in prehybridization buffer (50%

formamide 4x SSC, 50 mM sodium phosphate, pH 7.0, 100 μg/ml denatured salmon sperm DNA) for 2 h at 42°C prior to the addition of the denatured DNA probe (1 x 10^9 cpm/μg) that had been labeled with [α^{32}P]dCTP (Amersham) using the Prime-It II Random Primer Labeling Kit (Stratagene) according to the manufacturer's protocol. After further incubation at 42°C for 6 h, the membrane was washed once with 2x SSC-0.1% SDS for 10 min at room temperature and twice with the same buffer at 60°C for 15 min. The membrane was then exposed to x-ray film at -80°C. Hybridizing bands were quantitated by scanning of the x-ray film with a laser densitometer (Molecular Dynamics). Differences in sample loading were normalized by probing membranes with β-actin cDNA after the previous hybridization signals had been stripped from the membrane by incubation in 0.5% SDS at 90°C.

Reverse transcription-PCR- Reverse transcription reactions (100 μl) contained 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 40 units RNAsin (Promega), 2 pM random nonamers, 1 mM of each of the dNTPs, 20 units AMV reverse transcriptase (Boehringer Mannheim) and either total RNA (2 μg) or poly (A^+) RNA (0.2 μg). After incubation at 42°C for 45 min, reactions were terminated by heating at 94°C for 2 min. Aliquots (2 μl) that correspond to 40 ng of total RNA or 0.4 ng of poly (A^+) RNA were used as templates for amplification by the PCR. Amplifications by the PCR of sequences unique to the α and β species of mouse DNA ligase III cDNA were performed as described above except 5 μCi of [α^{32}P]dCTP was included in the reaction mixture. Amplifications from the same templates were carried out for 30 cycles of 94°C for 1.5 min, 60°C for 2 min and 72°C for 2 min using primers specific for mouse β-actin (Stratagene). After separation by electrophoresis through a 6% polyacrylamide gel, labeled PCR products in the dried gel were detected by autoradiography and quantitated by phosphorimage analysis (Molecular Dynamics).

The 30 cycles of amplification employed in the above experiments was determined to be within the log-linear range of amplification by quantitating PCR products as a function of the number of cycles of amplification. The RNA samples were free of detectable genomic DNA since the PCR amplifications using the β-actin primers, which reside in different exons, only

amplified a 514 bp product expected from the cDNA. Water blanks were also subjected to RT-PCR to test for target contamination in the assay reagents.

Expression of DNA ligase III fusion proteins- Human DNA ligase III cDNAs encoding the α and β forms of DNA ligase III were subcloned in-frame into pGSTag (30) to generate plasmids encoding glutathione-S-transferase (GST)-DNA ligase III fusion proteins. In addition, a truncated version of human DNA ligase III cDNA that encodes residues 1-700 was also subcloned in-frame into the same vector. After transformation into *E. coli* TG1, transformants were grown in TB media with 0.1 mg/ml ampicillin at 37°C. When the $O.D._{600}$ of the culture reached 0.6, IPTG was added to a final concentration of 1 mM and incubation was continued for 5 h at room temperature. Cells (1 liter) were harvested by centrifugation and resuspended in 10 ml of 50 mM HEPES-KOH (pH 8.0), 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM phenyl methylsulfonyl fluoride, 1 mM benzamidine. After lysis by sonication, the cleared lysate was incubated for 1 h at 4°C with 1 ml of glutathione sepharose 4B beads (Pharmacia Biotech) that had previously been washed with phosphate-buffered saline (PBS). The beads were poured into a column and washed with 10 ml of 50 mM HEPES-KOH (pH 8.0), 0.75 M NaCl, 1 mM EDTA, 1 mM dithiothreitol (buffer A). Bound proteins were eluted sequentially with 5 ml of buffer A containing 1 mM, 5 mM and 10 mM glutathione. The protein content of fractions (300 μl) was analyzed by Coomassie blue staining after denaturing gel electrophoresis (20). Fractions containing ATP-dependent DNA ligases were detected by the formation of labeled enzyme-adenylate intermediates when incubated with [α^{32}P]ATP (38). The GST-DNA ligase III fusion proteins, which were eluted by the 5 mM glutathione buffer, were aliquoted, flash-frozen in liquid nitrogen and stored at -80°C.

Preparation of DNA ligase III affinity resins- E. coli DH5α cells harboring plasmids that direct the expression of GST-DNA ligase III fusion proteins were grown in LB media with 0.1 mg/ml ampicillin as described above. Cells (500 ml culture) were harvested and resuspended in 5 ml of 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.1% nonidet P-40, 0.4 μg/ml aprotinin, 0.5 μg/ml chymostatin and leupeptin, 0.7 mg/ml pepstatin, 1 mM phenyl methylsulfonyl fluoride and 1 mM benzamidine (Buffer B) and lysed by sonication. After

clarification of the lysate by centrifugation, soluble proteins were mixed gently at 4°C for 1h with 100 µl of glutathione sepharose 4B beads (Pharmacia Biotech) that had previously been washed twice with PBS and once with 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% nonidet P-40, 0.5% dried milk. The beads were washed three times with buffer B and then stored on ice as a 50% slurry in buffer B. Aliquots (20 µl) from the different beads were incubated in SDS-sample buffer at 70°C for 10 min. After separation by denaturing gel electrophoresis, polypeptides were detected by staining with Coomassie blue. For each affinity bead, the major bound polypeptide corresponded to the expected size of the GST-DNA ligase III fusion proteins. A similar quantity of each of the GST-DNA ligase III fusion proteins was bound to the same volume of glutathione sepharose 4B beads.

Interaction between in vitro translated XRCC1 and DNA ligase III affinity resins- An EcoRI fragment containing *XRCC1* cDNA was subcloned from pcD2EX (7) into pBluescript KS (Stratagene). After the orientation of the EcoRI fragment had been determined by DNA sequencing, *XRCC1* was transcribed by T7 RNA polymerase and in vitro translated in the presence of [^{35}S] methionine using the Promega TNT system according to the manufacturer's protocol. Labeled XRCC1 was partially purified from the coupled in vitro transcription and translation reaction (50 µl) by ammonium sulfate precipitation (2) and resuspended in 50 µl of 50 mM Tris (pH 7.5), 0.1 M NaCl, 5 mM EDTA, 0.1 % nonidet P-40 (Buffer C). The DNA ligase III beads (30 µl) were washed three times with 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 % bovine serum albumin and then resuspended in 70 µl of buffer C prior to the addition of labeled XRCC1 protein (15 µl). After gentle mixing for 30 min at room temperature, the beads were washed five times with 1 ml of buffer A. SDS-sample buffer (30 µl) was added and samples were incubated at 70°C for 10 min to remove non-covalently attached proteins from the beads. After denaturing gel electrophoresis, the gel was soaked in Amplify (Amersham plc) as recommended by the manufacturer and then dried down. Labeled polypeptides were detected by fluorography and quantitated by scanning of the x-ray film with a laser densitometer (Molecular Dynamics).

Purification of recombinant XRCC1 protein- Recombinant XRCC1 protein with a poly (histidine) sequence at the C-terminus was purified from *E. coli* as described by Caldecott et al (8).

Far Western blotting analysis- GST-DNA ligase III fusion proteins were separated by denaturing gel electrophoresis and transferred to a nitrocellulose membrane. Polypeptides were renatured on the membrane as described (24). Recombinant XRCC1 protein was labeled and then incubated with the nitrocellulose membrane as described by Wei et al (42).

DNA ligase III antibodies- Mice were initially immunized with GST-DNA ligase III fusion protein (human DNA ligase III-β) bound to glutathione sepharose beads at two week intervals. Antiserum was collected 10 days after immunization. At later times, the mice were immunized with recombinant his-tagged mouse DNA ligase III (residues 44 to 868 of DNA ligase III-β) that had been purified by metal chelating affinity chromatography. The rabbit polyclonal antiserum specific for mammalian DNA ligase I has been described previously (21, 38).

Immunofluorescence- Mouse NIH 3T3 fibroblasts and HeLa S3 cells were grown in D-MEM (GibcoBRL) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin on 12 mm coverglass slips. Late pachytene spermatocytes purified from adult mouse testis were attached to poly-L-lysine coated glass slides. Attached cells were rinsed twice with PBS and then fixed for 3 minutes in ice cold methanol. After permeabilization by immersion in a solution of 0.25% Triton X-100 in PBS for 3 min at room temperature, cells were washed twice with PBS containing 0.5% BSA and then incubated with pre-immune or immune sera diluted in PBS containing 0.5% BSA at 37°C for 30 min in a humidified chamber. After being washed three times with PBS containing 0.5% BSA, the cells incubated for a further 30 min with the appropriate secondary antibodies linked either to rhodamine or fluorescein (Kirkegaard & Perry) that had been diluted 1:100 in PBS containing 0.5% BSA. Cells were then washed once with 0.5% BSA in PBS and twice with PBS. HeLa and NIH 3T3 cells were immersed in 4,6 diamino-2-phenolindole propidium iodide (DAPI, 1 mg/ml) for 1 min and then rinsed twice for 2 min in H_2O. Finally, coverslips were mounted to glass slides with permaFluore and examined on a Zeiss fluorescent microscope (magnification x1250).

Digital photographs were obtained with a Hamamatsu Photonics camera. Images were superimposed using Photoshop for Power MacIntosh software.

Results

Two forms of mammalian DNA ligase III cDNA- Human cDNAs encoding DNA ligase III have recently been reported by two different groups (9, 42). The nucleotide sequences of these cDNAs are identical except for the 3' ends. This divergence of nucleotide sequence results in the production of polypeptides that differ both in size and in amino acid sequence at their C-termini (Fig. 1). The cDNA isolated from the HeLa cDNA library, which encodes a polypeptide with a calculated molecular weight of 103,000, has been designated DNA ligase III-α. The cDNA isolated from the testis cDNA library, which encodes a polypeptide with a calculated molecular weight of 96,000, has been designated DNA ligase III-β. At the amino acid sequence level, the C-terminal 77 amino acid residues of DNA ligase III-α are replaced by an unrelated 17 amino acid sequence in DNA ligase III-β (Fig. 1). The differences in nucleotide sequence between these cDNAs may be the result of either a cloning artifact or alternative splicing. In support of the latter idea, the nucleotide sequences of the cDNAs at the site of sequence divergence resemble consensus splice donor/acceptor sequences (Fig. 1) and two species of DNA ligase III mRNA with estimated molecular masses of 3.4 and 3.6 kb have been observed (9).

If there are two alternatively spliced DNA ligase III mRNA species that encode polypeptides with distinct cellular functions, then one would expect this splicing event to be conserved in other mammals. In agreement with this hypothesis, we have isolated two species of DNA ligase III cDNA from a mouse testis cDNA library whose sequences diverge at the same place as, and exhibit homology with, the different 3' ends of human DNA ligase III cDNAs (Fig. 1). Human and mouse DNA ligase III-α cDNAs encode polypeptides consisting of 922 and 927 residues, respectively. These polypeptides exhibit about 90% amino acid identity overall and 86% within the 77 amino acid C-terminal region (Fig. 1A). Human and mouse DNA ligase III-β cDNAs encode polypeptides consisting of 862 and 868 residues,

respectively. These polypeptides also share about 90% amino acid identity overall but the degree of identity is only 60% within the different C-terminal region of 17-18 amino acids (Fig. 1B).

Genomic structure at the 3' end of the mouse DNA ligase III gene- To confirm that exons encoding the different C-termini of DNA ligase III-α and -β reside in the same region of the genome, we have cloned and analyzed an 18 kb fragment of mouse genomic DNA that contains the 3' end of the DNA ligase III gene. As expected, probes specific for the different 3' ends of the DNA ligase III cDNAs hybridize to distinct regions that have been localized to a 4.2 kb genomic fragment (Fig. 2A, 2B and 2C). The order and arrangement of exons and introns at the 3' end of the DNA ligase III gene shown in Figure 2D was determined by restriction enzyme mapping, Southern blotting (Fig. 2A, 2B and 2C) and DNA sequencing (Fig. 2E). The α and β species of DNA ligase III mRNA are produced by two different splices between the last common exon and two alternative terminal exons (Fig. 2D). The nucleotide sequences at the intron/exon junctions (Fig. 2E) exhibit homology with consensus splice donor and acceptor sequences (26).

Expression of DNA ligase III mRNAs in different mouse tissues- The steady state levels of DNA ligase III mRNAs in a variety of mouse tissues were examined by northern blotting. DNA ligase III mRNAs with estimated molecular masses of 3.4 and 3.6 kb were detected in the testis (Fig. 3A). Expression of DNA ligase III was detectable in all the other tissues after longer exposures (data not shown), indicating that, as in humans (9), DNA ligase III is ubiquitously expressed at a low level except in the testes where the steady state levels of DNA ligase III are at least 10-fold higher than in other tissues and cells.

We have used a more sensitive technique, reverse transcription (RT)-PCR, to investigate the expression of the alternatively spliced forms of DNA ligase III mRNA in different mouse tissues (Fig. 3B). In agreement with the northern blotting results, the highest levels of both forms of DNA ligase III mRNA were detected in the testis. The steady state level of DNA ligase III-α mRNA varies greatly in somatic tissues, ranging from 6% (spleen) to 55% (kidney) of the level in the testis. In contrast, the DNA ligase III-β mRNA is expressed at a

very low level in all the somatic tissues (<10%) compared with the level in the testis. The steady state levels of DNA ligase III-α and -β mRNAs in the ovary were similar to those in the liver. These RT-PCR studies in conjunction with the results from northern blotting experiments indicate that the 3.6 kb DNA ligase III-α mRNA is the predominant species in somatic tissues and cells. In the testis, both species of DNA ligase III mRNA are expressed at significantly higher levels than in somatic tissues with the 3.4 kb DNA ligase III-β being the predominant species in this tissue. Based on these observations, we conclude that DNA ligase III-β mRNA is generated by a testis-specific alternative splicing event.

DNA ligase III expression in the developing testis and in purified germ cells- In a previous study we have shown by northern blotting that expression of DNA ligase III correlates with the appearance and accumulation of meiotic cells in the developing mouse testis (9). Using RT-PCR, we have examined the expression patterns of the alternatively spliced forms of DNA ligase III mRNA during testis development (Fig. 4A). Expression of DNA ligase III-α mRNA was detectable in the testes of 5-day old animals and increased with age reaching a level in the testis of 25-day old animals that was about 3-fold higher than in the 5-day old animals. Although the highest steady state levels of DNA ligase III-β mRNA were also detected in the testis of 25-day old animals, expression of this mRNA species in the testis of the younger animals was barely detectable (<5% of the steady state levels in the testis of the 25-day old animal).

In the testis of a 25-day old mouse, the steady state level of 3.4 kb DNA ligase III-β mRNA is about 2-fold higher than the steady state level of 3.6 kb DNA ligase III-α mRNA (Fig. 4B). At this age, pachytene spermatocytes constitute about 30% of the cells in the seminiferous tubules (5). If, as suggested, the steady state levels of DNA ligase III mRNAs are significantly higher in this cell type compared with other cell types present in the testis then poly (A^+) RNA from pachytene spermatocytes should have a relatively higher level of DNA ligase III mRNA when compared with the same amount of poly (A^+) RNA from whole testis of 25-day old animals. In agreement with this hypothesis, we found that the steady state level of DNA ligase III-β mRNA is about 3-fold higher in late pachytene spermatocytes compared with whole

testes from a 25-day old animal. In contrast, DNA ligase III-α mRNA was present at similar levels in late pachytene spermatocytes and whole testis from a 25-day old animal (Fig. 4B).

Expression of the alternatively spliced forms of DNA ligase III mRNA has been examined by RT-PCR in purified testis germ cell populations representing different stages of spermatogenesis prior to spermiogenesis and in a purified somatic cell type, Sertoli cells, present in testis. Elevated expression of DNA ligase III-α mRNA in germ cells relative to the level in Sertoli cells was initially detected in pre-leptotene spermatocytes. The steady state levels continued to increase during meiotic prophase, reaching a peak in late pachytene spermatocytes. After the two meiotic divisions, the level of expression in round spermatids was similar to that observed in early pachytene spermatocytes (Fig. 4C and 4D). In contrast, elevated expression of DNA ligase III-β mRNA relative to the level in Sertoli cells was not detected until early pachytene. The steady state levels reached a peak in late pachytene spermatocytes and declined about 20% in round spermatids (Fig. 4C and 4D).

Interaction of DNA ligase III-α and DNA ligase III-β polypeptides with XRCC1- We have demonstrated that an alternative splicing event, which appears to be restricted to male germ cells from the latter stages of meiotic prophase to round spermatids, produces a form of DNA ligase III, DNA ligase III-β, that has a different C-terminus than the 103 kDa DNA ligase III-α which is present in both somatic and germ cells. The two forms of DNA ligase III have been expressed as GST-fusion proteins in *E. coli*. After affinity purification, both DNA ligase III-α and DNA ligase III-β fusion proteins formed the labeled enzyme-adenylate intermediate and there was no significant difference between the two forms in their ability to perform this reaction (Fig. 5A). This is consistent with the previous observations demonstrating that both the α and β forms of DNA ligase III are active as DNA joining enzymes (9, 42).

Although there are no apparent differences in the catalytic properties of the α and β forms of DNA ligase III, it is possible that the C-termini of these polypeptides are recognized by different proteins and it is these interacting proteins which determine the cellular function of the DNA ligase III isoforms. Since DNA ligase III has been shown to interact with XRCC1 (7,

8, 42), we have compared the binding of the α and β forms of DNA ligase III to XRCC1 by two different methods. In Figure 5B, equal amounts of GST-DNA ligase III-α and GST-DNA ligase III-β fusion proteins were separated by denaturing gel electrophoresis and transferred to a nitrocellulose membrane. After renaturation on the membrane, the GST-DNA ligase III fusion proteins were incubated with labeled XRCC1 protein. Consistent with previous studies (8, 42), XRCC1 formed a complex with the 103 kDa form of DNA ligase III-α. In contrast the amount of complex formed with DNA ligase III-β was reduced by greater than 10-fold.

To confirm the results obtained by far western blotting, in vitro translated XRCC1 protein was incubated with the GST-DNA ligase III fusion proteins bound to glutathione beads. Once again there was a significant difference in the interaction between XRCC1 and the different forms of DNA ligase III (Fig. 5C). Approximately 5-fold more XRCC1 bound to the glutathione beads with DNA ligase III-α as the ligand compared to glutathione beads with the same amount of DNA ligase III-β as the ligand. In similar experiments with either a GST-DNA ligase III fusion containing the amino terminal 700 residues of DNA ligase III (common to both DNA ligase III-α and DNA ligase III-β) or in the absence of a protein ligand, no binding of XRCC1 to the glutathione beads was detected (data not shown). These results demonstrate that interaction between DNA ligase III and XRCC1 requires the C-terminus of DNA ligase III and that the C-terminus of DNA ligase III-α binds to XRCC1 with significantly higher affinity than the C-terminus of DNA ligase III-β.

Intracellular distribution of DNA ligases I and III in mitotic and meiotic cells- We have examined the subcellular distribution of DNA ligases I and III in an unsynchronized, dividing population of a human cells by indirect immunofluorescence. In agreement with previous studies (21, 43), a punctate staining pattern was observed in the nuclei of cells in the DNA synthesis phase of the cell cycle with the DNA ligase I antiserum and when the nuclear membrane was absent during mitosis, DNA ligase I was dispersed throughout the cell (Fig. 6A) (21). In contrast, intense staining of the highly condensed chromatin present from metaphase to telophase was observed in both the human cells and mouse NIH 3T3 fibroblasts with the DNA

ligase III antiserum (Fig. 6A and 6B). No detectable staining of condensed chromatin was observed in similar experiments with pre-immune serum and DNA ligase III antiserum that had been pre-incubated with purified DNA ligase III (data not shown). In interphase cells, DNA ligase III was present throughout the nucleus except for the nucleolus and, in some of these cells, thin thread-like structures that may correspond to condensing chromosomes were stained by the DNA ligase III antiserum (Fig. 6A and 6B).

The subcellular distribution of DNA ligase III was examined in late pachytene spermatocytes purified from mouse testis. The highly condensed chromatin, which has been incorporated into synaptonemal complexes in these cells, was intensely stained by the DNA ligase III antiserum. (Fig. 6C). In similar experiments with DNA ligase I antiserum, the staining was dispersed throughout the nucleus of these cells. Thus, DNA ligase III associates with condensed chromatin in both mitotic and meiotic cells.

Discussion

Several examples of differences in gene expression between somatic and germs cells have been described, including different transcription initiation sites (14), differences in the length of the poly(A) tract (11, 16), alternative splicing (13) and the existence of homologous genes, one of which is expressed only in somatic cells while the other is only expressed in germ cells (17, 25). In this report we have characterized a testis-specific alternative splicing mechanism that generates a 96 kDa DNA ligase III polypeptide, DNA ligase III-β, with a different carboxy terminus and molecular mass than the ubiquitously expressed DNA ligase III-α mRNA,which encodes 103 kDa DNA ligase III-α. The conservation of this splicing mechanism in mammals suggests that it is biologically significant, possibly producing DNA joining enzymes that are required for distinct cellular functions.

DNA ligase III has been implicated in the repair of DNA damage introduced by alkylating agents and ionizing radiation by virtue of it's association with the human DNA repair protein XRCC1 (7, 8, 36, 37, 42). This suggests that DNA ligase III may be involved in DNA base excision repair and in the repair of DNA single-strand breaks. We have found that XRCC1

interacts with 103 kDa DNA ligase III-α but has little affinity for 96 kDa DNA ligase III-β. This result demonstrates that the C-terminal 77 residues of DNA ligase III-α are required for the high affinity interaction with XRCC1. The high degree of amino acid identity shared between human and mouse DNA ligase III-α in this region presumably reflects the functional significance of the interaction with XRCC1 and provides a plausible explanation for the observed interaction between human XRCC1 and chinese hamster DNA ligase III (7). However, the inability of DNA ligase III-β to interact with XRCC1 suggests that this form of DNA ligase III participates in a cellular function(s) distinct from those involving the 103 kDa DNA ligase III-α·XRCC1 complex.

In an attempt to gain insight into the biological function of DNA ligase III-β, the expression of DNA ligase III-β mRNA has been examined as a function of male germ cell differentiation and compared with the expression of DNA ligase III-α mRNA. Since late pachytene spermatocytes and round spermatids were the only cell types with high steady state levels of DNA ligase III-β mRNA, the alternative splicing mechanism appears to be turned on during the latter stages of meiotic prophase. Although there are other possibilities, the specific regulation of this splicing event strongly suggests that DNA ligase III-β is involved in the completion of meiotic recombination events at the end of the pachytene stage or during the diplotene stage of meiotic prophase prior to the first meiotic cell division.

Although the highest steady state levels of DNA ligase III-α mRNA are also present in late pachytene spermatocytes, increased levels of this species of mRNA occur earlier in meiotic prophase. This expression pattern is essentially the same as that observed for the *XRCC1* gene (40, 41), whose product interacts with DNA ligase III-α in somatic cells (7, 8, 42). This apparent co-ordinate regulation suggests that the 103 kDa DNA ligase III-α·XRCC1 complex is also present in male germ cells and presumably functions in the same DNA repair pathways in these cells as it does in somatic cells. DNA repair, as measured by unscheduled DNA synthesis, occurs in round spermatids (19). Furthermore, these cells contain high levels of the DNA base excision repair enzyme, DNA polymerase β (1). Thus it appears that the capacity to carry out at

least some forms of DNA repair is retained and may be even enhanced in meiotic and early post-meiotic germ cell types.

DNA ligase III associates with condensed chromatin in both mitotic and meiotic cells. Although it is possible that this association occurs solely to ensure the equal distribution of DNA ligase III to the daughter cells, we suggest that DNA ligase III also binds to chromatin in interphase cells and that this interaction is necessary for DNA ligase III to join DNA single-strand breaks in DNA repair and meiotic recombination pathways. The predicted amino acid sequence of DNA ligase III contains a region near the amino terminus that is homologous to a DNA-binding zinc finger that has been characterized in poly(ADP) ribose polymerase (10). This putative zinc finger of DNA ligase III may be involved in the interaction with chromatin. Alternatively, the interaction of DNA ligase III with chromatin may be mediated by protein-protein interactions. Based on the uniform staining of metaphase chromosomes in mitotic cells by the DNA ligase III antiserum, it seems likely DNA ligase III-α will bind to condensed chromatin in meiotic cells and thus, will be distributed along the entire length of the synaptonemal complexes that occur in the latter stages of meiotic prophase. If DNA ligase III-β is specifically involved in the completion of meiotic recombination events, it may be localized at discrete sites along the synaptonemal complex as has been observed for the eukaryotic recombination protein, RAD51 (6, 15).

In summary we have shown that an alternative splicing mechanism, which is uniquely activated in male meiotic cells, produces DNA ligase III-β that can be distinguished from DNA ligase III-α by it's inability to interact with the DNA repair protein, XRCC1. We suggest that DNA ligase III-β plays a specific role in the completion of the numerous homologous recombination events which occur during meiotic prophase. In contrast the mRNA encoding DNA ligase III-α is expressed in both somatic and germ cells, suggesting that the DNA ligase III-α·XRCC1 complex functions in DNA repair in all cell types. The development of immunological reagents that distinguish between the α and β forms of DNA ligase III will

facilitate further investigations into the cellular functions involving DNA ligase III in somatic and germ cells.

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Figure Legends

Figure 1. Alignment of the carboxy terminal amino acid sequences encoded by the different 3' nucleotide sequences of mammalian DNA ligase III cDNAs

Human DNA ligase III cDNA sequences that differ at their 3' end have been reported previously (9, 42). Nucleotide and amino acid residues are numbered on the left. **A.** Alignment of the carboxy terminal sequences of the 922 and 927 amino acid polypeptides encoded by human and mouse DNA ligase III-α cDNAs, respectively. **B.** Alignment of the carboxy terminal sequences of the 862 and 868 amino acid polypeptides encoded by human and mouse DNA ligase III-β cDNAs, respectively. The nucleotide residues at the site where the cDNA sequences of mammalian DNA ligase III diverge are underlined. Consensus splice donor and acceptor sequences are shown.

Figure 2. Structural organization and restriction map at the 3' end of the mouse *LIG3* gene

A genomic fragment containing the 3' end of the mouse *LIG3* gene was isolated as described in Materials and Methods. A 4.2 kb BamH I fragment of genomic DNA was subcloned into pBluescript KS and the resultant plasmid digested with Apa I, EcoR V, Kpn I and Pst I as indicated. A 1.3 kb cDNA fragment corresponding to the 3' end of mouse DNA ligase III-α cDNA was subcloned into pBluescript KS and the resultant plasmid was digested with EcoR I. **A.** Ethidium bromide stained agarose gel. After transfer of the DNA fragments to nitrocellulose membranes, the membranes were hybridized with probes specific for the different 3' ends of mouse DNA ligase III cDNA. **B.** 425 bp probe specific for mouse DNA ligase III-α cDNA. **C.** 150 bp probe specific for mouse DNA ligase III-β cDNA. **D.** Restriction map of the 4.2 kb genomic fragment. The position of the last common exon shared by the α and β versions of mouse DNA ligase III cDNA is indicated by the boxed region, C. The boxed region, β, corresponds to the unique coding sequence at the 3' end of DNA ligase III-β cDNA and the boxed region, α, corresponds to the unique coding sequence at the 3' end of DNA ligase III-α

cDNA. The splicing events that generate the two species of DNA ligase III cDNA are indicated. **E.** Nucleotide sequences at the intron/exon junctions of exons C, β and α.

Figure 3. Tissue and cell distribution of mouse DNA ligase III mRNAs.

A. A northern blot containing ~2 μg of poly $(A)^+$ RNAs from various mouse tissues Northern blotting was sequentially hybridized with a 0.9-kb mouse DNA ligase III cDNA fragment (nucleotides 1448-2333) and a 2 kb human β-actin cDNA fragment. **B.** Expression of the α and β forms of mouse DNA ligase III cDNA in various mouse tissues was examined by RT-PCR. Reverse transcription reactions and PCR amplifications were performed with total RNA as described in Materials and Methods. PCR products were separated by polyacrylamide gel electrophoresis and detected by autoradiography. The 425 bp PCR product amplified by the primers specific for DNA ligase III-α cDNA, the 150 bp PCR product amplified by the primers specific for DNA ligase III-β cDNA and the 514 bp PCR product amplified by primers specific for mouse β-actin are indicated.

Figure 4. Steady state levels of DNA ligase III mRNAs during mouse testis development and in germ cell populations purified from mouse testis.

A. Expression of the α and β forms of mouse DNA ligase III cDNA in the testis of 5-, 15-, 25- and 83-day old mice. RT-PCR was performed with poly $(A)^+$ RNA samples isolated from the testes of different aged mice as described in Materials and Methods. PCR products were separated by polyacrylamide gel electrophoresis and detected by autoradiography. The 425 bp PCR product amplified by the primers specific for DNA ligase III-α cDNA, the 150 bp PCR product amplified by the primers specific for DNA ligase III-β cDNA and the 514 bp PCR product amplified by primers specific for mouse β-actin are indicated. **B.** Poly $(A)^+$ RNA samples from the testis of a 25-day old mice and from late pachytene spermatocytes were electrophoresed through a 1.2% agarose-formaldehyde gel and then transferred to a nitrocellulose membrane as described in Materials and Methods. The membrane was sequentially hybridized with a 0.9-kb mouse DNA ligase III cDNA fragment (nucleotides 1448-

2333) and a 2 kb human β-actin cDNA fragment. **C.** RT-PCR was performed with total RNA samples isolated from different testis cell types as described in Materials and Methods. PCR products were separated by polyacrylamide gel electrophoresis and detected by autoradiography. The 425 bp PCR product amplified by the primers specific for DNA ligase III-α cDNA, the 150 bp PCR product amplified by the primers specific for DNA ligase III-β cDNA and the 514 bp PCR product amplified by primers specific for mouse β-actin are indicated. **D.** Graphic representation of the data shown in panel C. After quantitation of the PCR products by phosphorimage analysis, the values obtained for each of the DNA ligase III cDNA products were divided by the value obtained for the β-actin cDNA product. Since the highest expression ratios for both the α and β forms of DNA ligase III cDNA were obtained in late pachytene spermatocytes, the expression ratios in the other cell types are expressed as a percentage of the ratio in late pachytene spermatocytes. Shaded bar, DNA ligase III-α; filled bar, DNA ligase III-β.

Figure 5. Interaction of DNA ligase III-α and DNA ligase III-β with XRCC1. **A.** GST-DNA ligase III fusion proteins (10 ng) were adenylylated as described in Materials and Methods. After separation by denaturing gel electrophoresis, labeled polypeptides in the dried gel were detected by autoradiography. Lanes containing the adenylylated GST-DNA ligase III fusion proteins, which have an estimated molecular mass of 125 kDa, are indicated by α and β. **B.** GST-DNA ligase III fusion proteins (100 ng) were separated by denaturing gel electrophoresis and transferred to nitrocellulose membranes. After renaturation, membrane-bound polypeptides were incubated with ^{32}P-labeled XRCC1 as described in Materials and Methods. Labeled complexes were detected by autoradiography. Lanes labeled α and β contain GST-DNA ligase III-α and GST-DNA ligase III-β proteins, respectively. **C.** GST-DNA ligase III fusion proteins bound to glutathione sepharose beads were incubated with labeled, in vitro translated XRCC1 as described in Materials and Methods. Labeled polypeptides that bound to the beads were detected by fluorography after separation by denaturing gel electrophoresis.

Lanes labeled α and β correspond to experiments with GST-DNA ligase III-α beads and GST-DNA ligase III-β beads, respectively. Lane X contains in vitro translated XRCC1. The positions of ^{14}C-labeled molecular mass standards (Amersham plc) are shown on the left.

Figure 6. Intracellular localization of DNA ligases I and III in human and mouse somatic cells and in mouse pachytene spermatocytes by indirect immunofluorescence.

After fixation, cells were incubated with a rabbit polyclonal antiserum specific for DNA ligase I (LIG I, 1:1000 dilution) and a mouse polyclonal antiserum specific for DNA ligase III (LIG III, 1:1000 dilution) as described in Materials and Methods. Staining with DNA ligase I antibody (LIG I) was detected by indirect immunofluorescence using a rhodamine-conjugated secondary antibody (red fluorescence). Staining with DNA ligase III antibody (LIG III) was detected by indirect immunofluorescence using a fluorescein-conjugated secondary antibody (green fluorescence). **A.** HeLa S3 cells. DAPI staining detected by direct fluorescence. The same microscopic field analyzed by indirect immunofluoresnce with the indicated antibody. **B.** Mouse NIH 3T3 fibroblasts. DAPI staining detected by direct fluorescence. The same microscopic field analyzed by indirect immunofluorescence with the indicated antibody. **C.** Mouse pachytene spermatocytes. In the first panel, cells visualized by phase contrast microscopy. The same microscopic field analyzed by indirect immunofluorescence with the indicated antibody.

A.

Splice donor/acceptor consensus sequences
5' exon C/AAG G/A 3' exon

```
        846     P   C   L   K   K   V   L   L   D   V   F   T   G   V   R   L   Y   L
Mouse  2897     CCA TGC CTG AAA AAG GTA CTG TTG GAT GTC TTC ACT GGG GTG CGG CTC TAC TTG

Human  2872     CTG TGC CAA ACA AAG GTA TTG CTG GAC ATC TTC ACT GGG GTG CGG CTT TAC TTG
        841     L   C   Q   T   K   V   L   L   D   I   F   T   G   V   R   L   Y   L

        864     P   P   S   T   P   D   F   K   R   L   K   R   Y   F   V   A   F   D
Mouse  2951     CCA CCT TCT ACA CCA GAC TTC AAA CGT CTC AAA CGC TAC TTT GTG GCA TTC GAC

Human  2926     CCA CCC TCC ACA CCA GAC TTC AGC CGT CTC AGA CGC TAC TTT GTG GCA TTC GAC
        859     P   P   S   T   P   D   F   S   R   L   R   R   Y   F   V   A   F   D

        882     G   D   L   V   Q   E   F   D   M   G   S   A   T   H   V   L   G   N
Mouse  3005     GGG GAC CTG GTA CAG GAA TTT GAC ATG GGC TCA GCC ACA CAT GTG CTA GGT AAC

Human  2980     GGG GAC CTG GTA CAG GAA TTT GAT ATG ACT TCA GCC ACG CAC GTG CTG GGT AGC
        877     G   D   L   V   Q   E   F   D   M   T   S   A   T   H   V   L   G   S

        900     R   E   K   N   T   D   A   Q   L   V   S   S   E   W   I   W   A   C
Mouse  3059     AGG GAA AAG AAC ACT GAT GCC CAG TTG GTC TCC TCA GAG TGG ATT TGG GCA TGT

Human  3034     AGG GAC AAG AAC CCT GCG GCC CAG CAG GTC TCC CCA GAG TGG ATT TGG GCA TGT
        895     R   D   K   N   P   A   A   Q   Q   V   S   P   E   W   I   W   A   C

        918     I   R   K   R   R   L   I   A   P   C   *
Mouse  3113     ATC CGG AAA CGG AGG CTG ATA GCT CCC TGC TAG GAC TTT GGT CTT CCT

Human  3088     ATC CGG AAA CGG AGA CTG GTA GCT CCC TGC TAG GTT TGC TGT CTT CCC
        913     I   R   K   R   R   L   V   A   P   C   *
```

Mackey et al, Fig. 1A

B.

Splice donor/acceptor consensus sequences
5' exon C/AAG G/A 3' exon

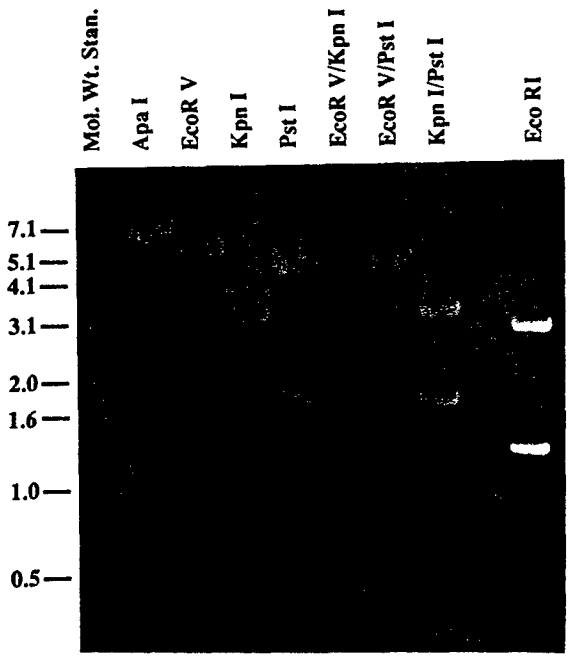
```
        846     P   C   L   K   K   R   R   R   A   S   R   Q   R   G   R   K   A   M
Mouse  2897     CCA TGC CTG AAA AAG AGG CGG CGG GCC AGC CGG CAA AGA GGA AGG AAA GCT ATG

Human  2872     CTG TGC CAA ACA AAG AGG CGG CCA GCC AGT GAG CAG AGA GGA AGA ACT GTG CCA
        841     L   C   Q   T   K   R   R   P   A   S   E   Q   R   G   R   T   V   P

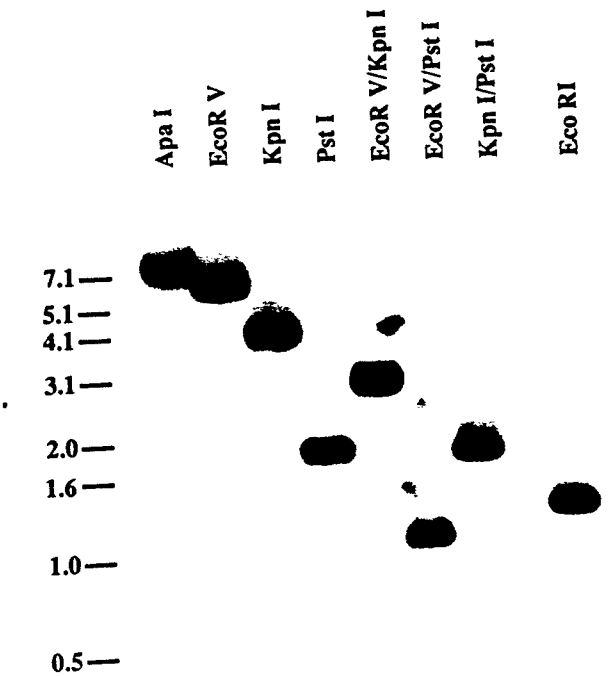
        864     Q   T   G   R   R   *
Mouse  2951     CAG ACA GGC AGG AGA TAG AGC CGG

Human  2926     GCA GGC AGG AGA TAG AAC AGC CCG
        859     A   G   R   R   *
```

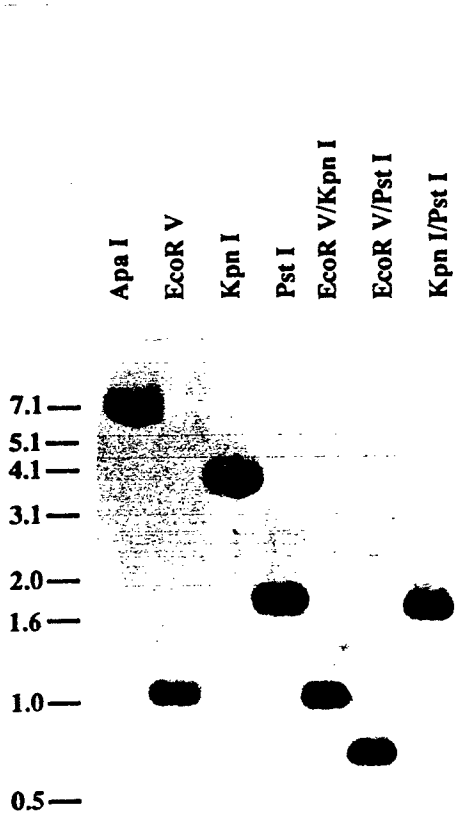
Mackey et al, Fig. 1B



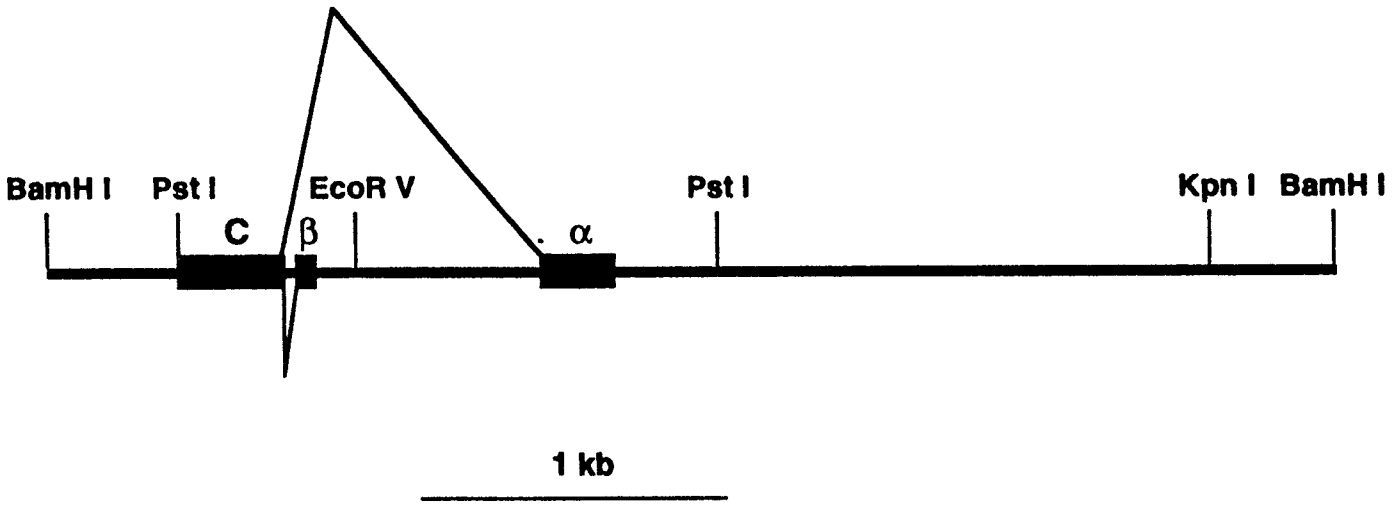
Mackey et al, Fig. 2A



Mackey et al, Fig. 2B



Mackey et al, Fig. 2C



Mackey et al, Fig. 2D

Exon C

```
            A   S   N       L   K   K
----ctgcag GCA TCA AAC ----CTG AAA AAG gttgag----
```

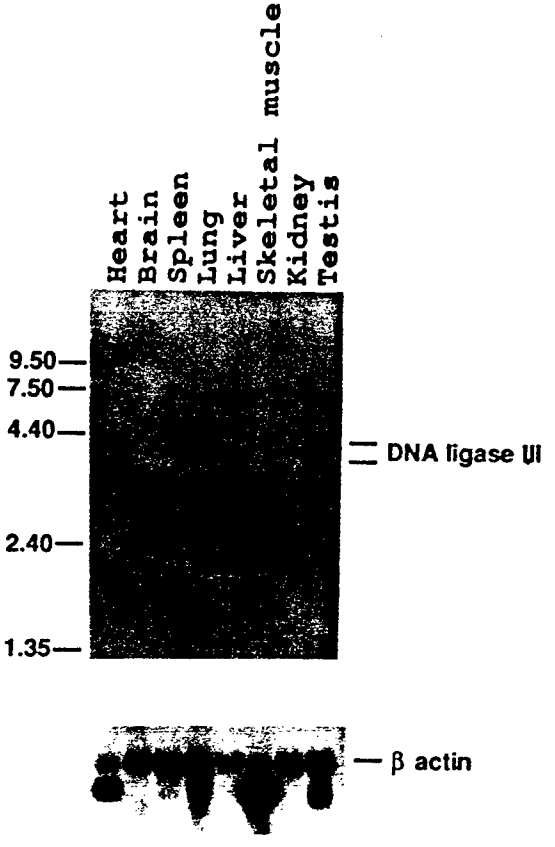
Exon β

```
            R   R   R       R   R   *
----ttgcag AGG CGG CGG ----AGG AGA TAG agccgg----
```

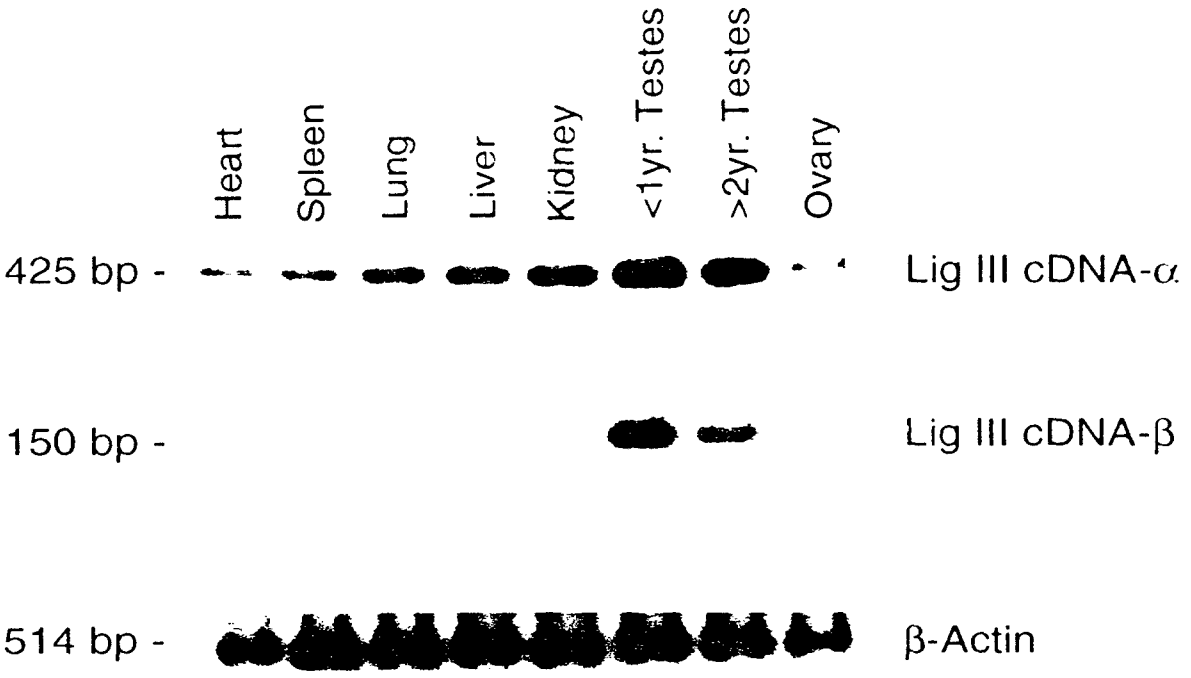
Exon α

```
            V   L   L       P   C   *
----ttgcag GTA CTG TTG ----CCC TGC TAG gacttt----
```

Figure 2E

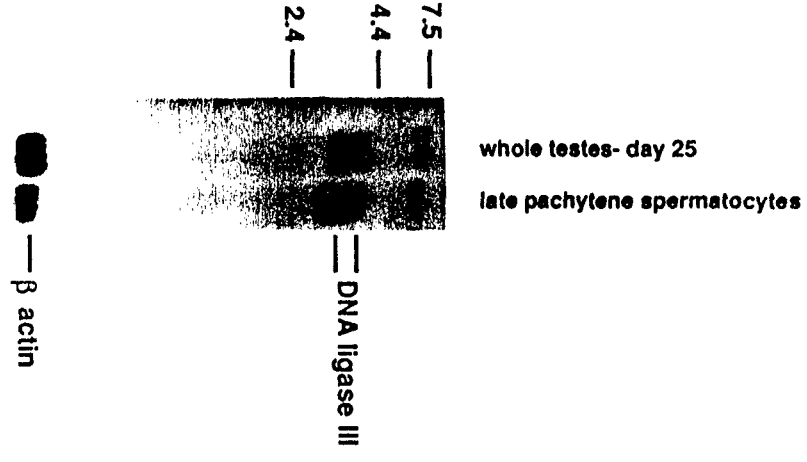


Mackey et al, Fig. 3A



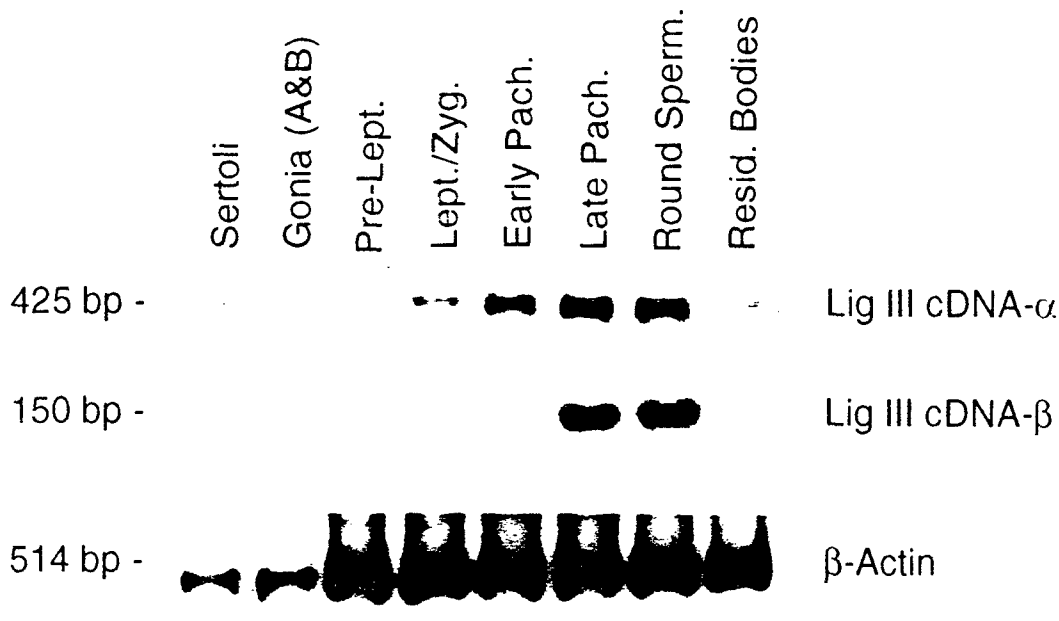
Mackey et al, Fig. 3B

Mackey et al, Fig. 4A



Mackey et al, Fig. 4B

Mackey et al, Fig. 4C



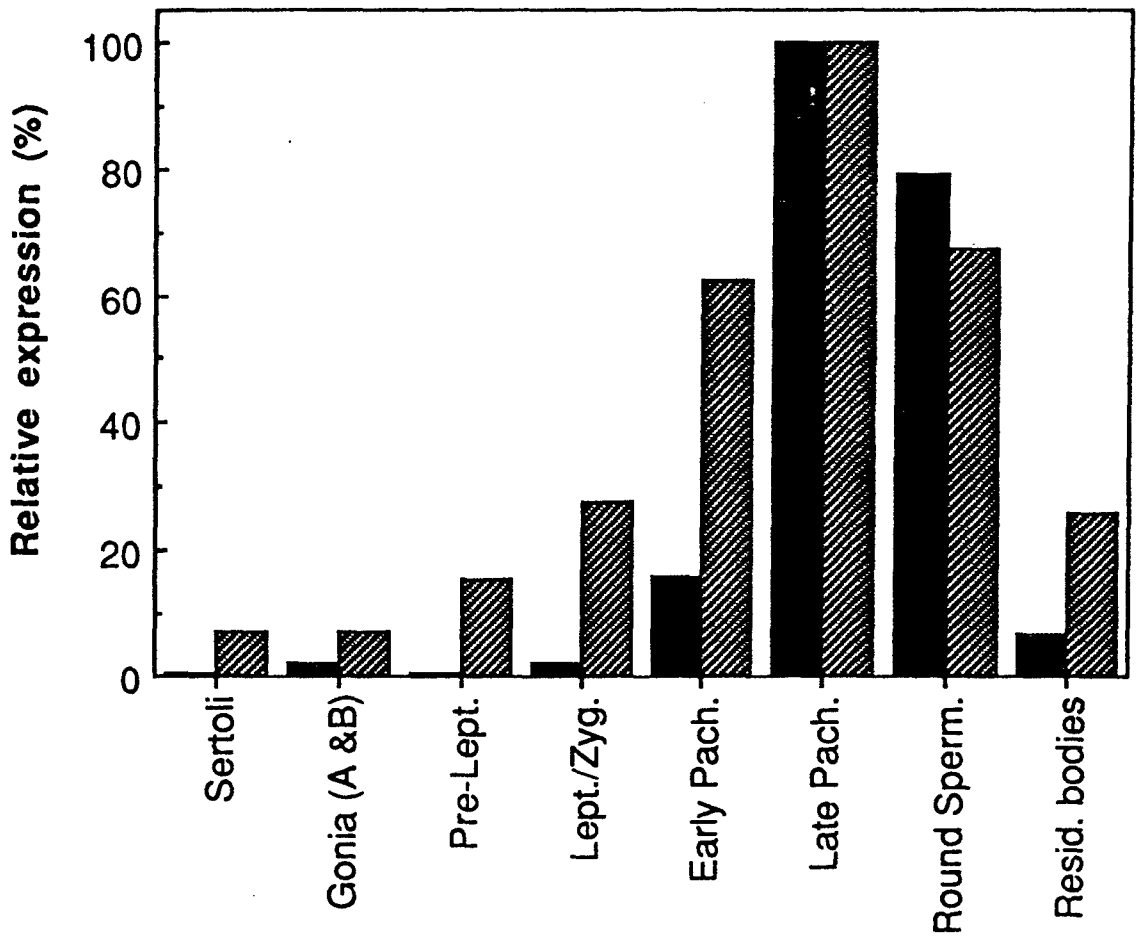
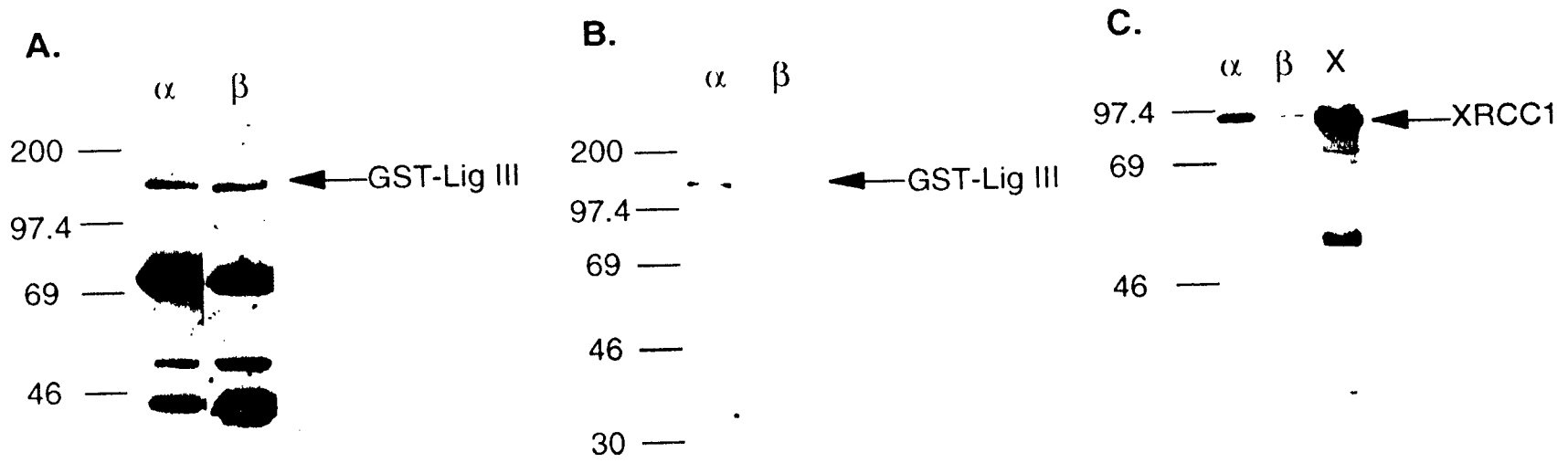

Relative expression (%)
100
80
60
40
20
0
Sertoli
Gonia (A &B)
Pre-Lept.
Lept./Zyg.
Early Pach.
Late Pach.
Round Sperm.
Resid. bodies

Figure 4D



Mackey et al, Fig. 5

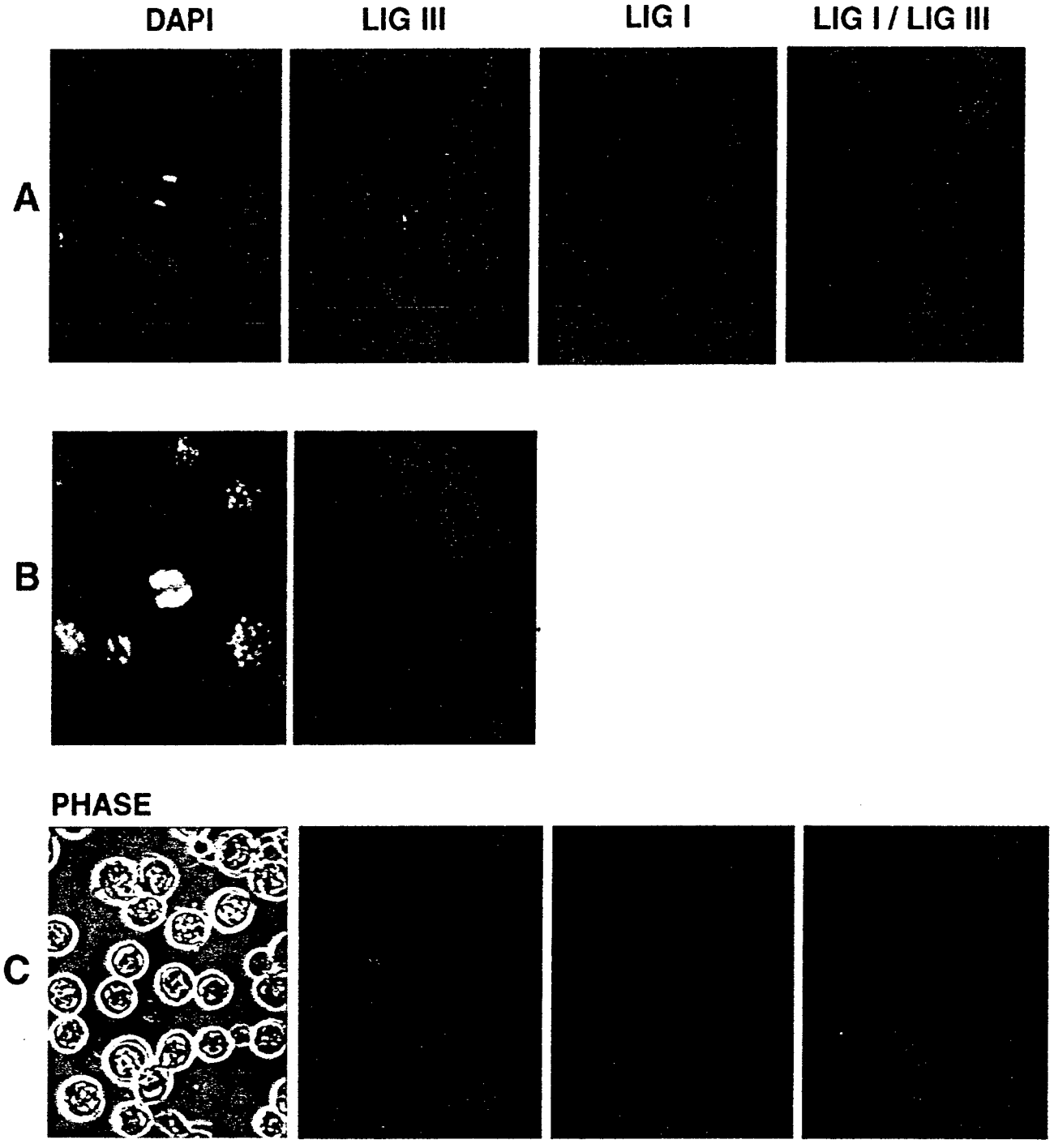


Figure 6

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Mammalian DNA Ligase II Is Highly Homologous with Vaccinia DNA Ligase

IDENTIFICATION OF THE DNA LIGASE II ACTIVE SITE FOR ENZYME-ADENYLATE FORMATION*

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Mammalian cells contain three biochemically distinct DNA ligases. In this report we describe the purification of DNA ligase II to homogeneity from bovine liver nuclei. This enzyme interacts with ATP to form an enzyme-AMP complex, in which the AMP moiety is covalently linked to a lysine residue. An adenylylated peptide from DNA ligase II contains the sequence, Lys-Tyr-Asp-Gly-Glu-Arg, which is homologous to the active site motif conserved in ATP-dependent DNA ligases. The sequences adjacent to this motif in DNA ligase II are different from the comparable sequences in DNA ligase I, demonstrating that these enzymes are encoded by separate genes. The amino acid sequences of 15 DNA ligase II peptides exhibit striking homology (65% overall identity) with vaccinia DNA ligase. These peptides are also homologous (31% overall identity) with the catalytic domain of mammalian DNA ligase I, indicating that the genes encoding DNA ligases I and II probably evolved from a common ancestral gene. Since vaccinia DNA ligase is not required for DNA replication but influences the ability of the virus to survive DNA damage, the homology between this enzyme and DNA ligase II suggests that DNA ligase II may be involved in DNA repair.

DNA joining is an essential step in the replication, repair, and recombination of the mammalian genome. This function appears to be fulfilled by three biochemically distinct DNA ligases (1), although whether these enzymes are encoded by separate genes remains controversial. At the present time, only the gene encoding DNA ligase I has been cloned and sequenced (2, 3). The predicted amino acid sequence of the catalytic domain of this enzyme exhibits approximately 50% identity with the DNA ligases encoded by the *CDC9* gene of *Saccharomyces cerevisiae* and the *Cdc17*$^+$ gene of *Schizosaccharomyces pombe* and about 30% identity with the DNA ligase encoded by vaccinia virus (2, 4–6). Genetic and biochemical studies on mammalian DNA ligase I, Cdc9 DNA ligase and Cdc17 DNA ligase have demonstrated that these functionally homologous enzymes are required for the joining of Okazaki fragments during DNA replication and are also involved in DNA repair (2, 7–10). Inactivation of the vaccinia DNA ligase gene has no significant effect on the replication and recombination of this cytoplasmic virus but the mutated virus is less virulent and more sensitive to DNA damage (11, 12).

Considerably less is known about the cellular functions of DNA ligases II and III. DNA ligase II was initially described as a labile, minor activity in calf thymus extracts (13). This 70-kDa polypeptide has a different substrate specificity than DNA ligase I, in particular the ability to join oligo(dT) molecules hybridized to a poly(rA)[1] template (14), and is not recognized by a polyclonal antiserum raised against homogeneous DNA ligase I (15, 16). Unlike DNA ligase I, which is required for DNA replication, DNA ligase II appears to be present at similar levels in proliferating and non-dividing tissues. In a normal mammalian liver, DNA ligase II is the predominant activity, contributing about 80% of the total cellular DNA joining activity (17, 18). Increases in the level of DNA ligase II activity have been observed following DNA damage, suggesting that this enzyme may be involved in DNA repair (19, 20).

A second minor DNA ligase activity, which is also active on the oligo(dT)·poly(rA) substrate, has been partially purified from calf thymus extracts (1). Based on differences in chromatographic, physical, and catalytic properties, this activity was designated DNA ligase III (1). Recently, the 100-kDa DNA ligase III has been identified as a component of a 500-kDa recombination complex purified from calf thymus glands (21) and as a component of a complex that contains the product of the human *XRCC1* gene, which appears to be involved in the repair of DNA single-strand breaks (22).

In this report we describe the purification of DNA ligase II to apparent physical homogeneity from bovine liver. The amino acid sequence of several DNA ligase II peptides, including a peptide that contains the active site lysine residue, has been determined. Although the sequence of the adenylylated DNA ligase II peptide contains the DNA ligase active site motif, Lys-Tyr/Ala-Asp-Gly-Xaa-Arg, that was originally identified in bovine DNA ligase I (23), the sequence adjacent to this motif is clearly different from the corresponding region of bovine DNA ligase I. A comparison of DNA ligase II peptide sequences with other eukaryotic DNA ligases demonstrated that this enzyme is more highly related to the DNA ligase encoded by vaccinia DNA ligase than to mammalian DNA ligase I and other replicative DNA ligases.

MATERIALS AND METHODS

Preparation of DNA Ligase Substrates—Oligonucleotide $(dT)_{16}$ and polynucleotides (dA), (rA), and (dT) were purchased from Pharmacia

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[1] The abbreviations used are: poly(rA), polyadenylic acid; FPLC, fast protein liquid chromatagraphy; HPLC, high pressure liquid chromatography; PLP, pyridoxal 5′-phosphate.

Biotech Inc. Labeled homopolymer DNA substrates and a substrate comprising of an end-labeled 16-mer, an 18-mer, and a complementary 34-mer, which form a duplex structure containing a single, internal nick, were prepared as described previously (24).

DNA Ligase Assays—Phosphodiester bond formation was assayed as described previously (24). One unit of DNA ligase activity catalyzes the conversion of 1 nmol of terminal phosphate residues to a phosphatase-resistant form in 15 min at 16 °C.

Analysis of Ligation Products—Aliquots (10 µl) from DNA ligase assays were added to 10 µl of formamide dye and heated for 2 min at 80 °C. Samples (2.5 µl) were then loaded onto a denaturing 10% polyacrylamide gel. After electrophoresis, the gels were dried, and oligonucleotides were visualized by autoradiography.

Formation of DNA Ligase-Adenylate Complex—Adenylylation reactions were carried out as described previously (25). The reactions were incubated at room temperature for 15 min and stopped by the addition of SDS sample buffer. After heating at 90 °C for 10 min, proteins were separated by SDS-polyacrylamide gel electrophoresis (26). Gels were fixed for 10 min in 10% acetic acid, dried, and exposed to x-ray film.

Purification of Bovine DNA Ligase II—Bovine livers were obtained from newly slaughtered cows at the local abattoir. The tissues were packed in ice and used for subcellular fractionation within 3 h. Nuclei were isolated from a liver (~5 kg) as described previously (15, 27) and stored as pellets at −80 °C. Nuclei (corresponding to 2.5 kg of tissue) were resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 0.3 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.4 µg/ml aprotinin, 0.5 µg/ml chymostatin, 0.5 µg/ml leupeptin, and 0.7 µg/ml pepstatin (buffer A) and lysed by Dounce homogenization. After 1 h on ice, the suspension was centrifuged at 40,000 rpm for 90 min in a Sorvall T-647.5 rotor. The nuclear extract (660 ml, 3.5 g) was fractionated by ammonium sulfate precipitation (15). The 45–70% ammonium sulfate precipitate was resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol containing the same protease inhibitors as buffer A (buffer B) and dialyzed against buffer B. After removal of insoluble debris by centrifugation, the dialysate (2.18 g, 300 ml) was applied to a 200-ml phosphocellulose column. Bound proteins were eluted from the column with a 2-liter linear gradient from 50 mM to 0.75 M NaCl in buffer B. Fractions were assayed for DNA joining and formation of the enzyme-AMP complex. Two peaks of DNA ligase activity were detected in assays with the oligo(dT)·poly(dA) substrate. A minor peak, which eluted at 0.15 M NaCl, contained the 125-kDa DNA ligase I. The major peak, which eluted at about 0.4 M NaCl, contained the 70-kDa DNA ligase II. Ligation of the oligo(dT)·poly(rA) substrate was detected only in assays with fractions from the major peak. Fractions from this peak were pooled (200 ml, 40 mg) and adjusted to 1 mM potassium phosphate (pH 7.5) prior to loading onto a 10-ml hydroxylapatite column that had been pre-equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 M NaCl, 10% glycerol, 1 mM potassium phosphate (pH 7.5). Proteins were eluted stepwise from the column with 50, 150, and 400 mM potassium phosphate (pH 7.5) buffers containing 10 mM 2-mercaptoethanol and 10% glycerol. DNA ligase II activity was detected in the 400 mM potassium phosphate eluate (22 ml, 14 mg). After dialysis against 25 mM Hepes-NaOH (pH 7.6), 1 mM EDTA, 100 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, 0.2% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine (buffer C), the sample was applied to a 3-ml double-stranded DNA cellulose column. Bound proteins were eluted stepwise with 0.2 and 0.5 M NaCl in buffer C. DNA ligase II, which was present in the 0.5 M NaCl eluate (4 ml, 0.6 mg), was loaded onto a 1.6 × 60-cm Superdex 200 column (Pharmacia). After elution with buffer C containing 0.5 M NaCl, DNA ligase II containing fractions (12 ml, 0.15 mg) were dialyzed against buffer C and loaded onto an FPLC Mono Q HR5/5 column. DNA ligase II activity, which was detected in the pass-through fractions (14 ml, 0.1 mg), was loaded onto an FPLC Mono S HR5/5 column. Bound proteins were eluted with a 20-ml linear gradient from 100 to 500 mM NaCl in buffer C without Tween 20. DNA ligase II fractions (1 ml, 20 µg), which eluted at 250 mM NaCl, were aliquoted and stored at −80 °C.

Purification of Recombinant Human DNA Ligase I—Human DNA ligase I cDNA was subcloned into a baculovirus expression vector, pVL1392 (PharMingen). The details of the purification of recombinant human DNA ligase I from baculoviral infected insect cells will be described elsewhere. In assays with the oligo(dT)·poly(dA) substrate, homogeneous 125-kDa DNA ligase I had a specific activity of 2.5 units/mg.

Inhibition of DNA Ligase II-Adenylate Formation by Pyridoxal 5′-Phosphate—PLP (Sigma) was dissolved in 25 mM Hepes-NaOH (pH 7.6) to 10 mM immediately prior to use. Bovine DNA ligase II (40 ng, 0.6 pmol) was incubated in reaction mixtures (10 µl) containing 25 mM Hepes-NaOH (pH 7.6), 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM dithiothreitol, and 0, 50, 100, or 200 µM PLP at 30 °C. After 5 min, 5 µl of 25 mM Hepes-NaOH (pH 7.6) containing 2 µCi of [α^{32}P]ATP (3000 Ci/mmol) were added, and incubation was continued for 15 s. The reactions were stopped by freezing in liquid nitrogen. It was not necessary to convert the Schiff's base formed between PLP and lysine to a covalent bond with sodium borohydride because of the short incubation for the adenylylation reaction. After separation by denaturing gel electrophoresis as described above, enzyme-adenylate complexes were detected by autoradiography and quantitated by phosphorimage analysis (Molecular Dynamics).

Immunoblotting—Proteins were separated by denaturing polyacrylamide gel electrophoresis (26) and transferred to nitrocellulose. After incubation with either antiserum raised against homogeneous bovine DNA ligase I (25) or antiserum raised against the conserved COOH-terminal peptide of eukaryotic DNA ligases (25), antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Corp.).

Proteolytic Digestion and Amino Acid Sequencing of Bovine DNA Ligase II Peptides—DNA ligase II (20 µg) was applied to a hydrophobic sequencing column (Hewlett-Packard) according to the manufacturers instructions. After *in situ* digestion with endoproteinase Lys-C (Wako), peptides were separated by reverse phase HPLC using a Spheri 5 ODS (Brownlee) column (28). The amino acid sequences of peptides were determined by automated Edman degradations performed on the ABI477A protein sequencer with the 120A phenylthiohydantoin analyzer.

Isolation of an Adenylylated Peptide from Bovine DNA Ligase II—DNA ligase II (20 µg, 290 pmol) was incubated with 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 4 µM ATP, and 100 µCi of [2,5′,8-^{3}H]ATP for 15 min at 25 °C. The reaction was terminated by the addition of EDTA (pH 8.0) to a final concentration of 10 mM. Protein was digested as described above, and the resultant peptides were separated by reverse phase HPLC on a C_8/RP300 (Brownlee) column as described previously (23). The adenylylated peptide was detected by monitoring absorbency at 214, 260, and 280 nm and by liquid scintillation counting. Peptides were sequenced as described above.

RESULTS

Purification of Bovine DNA Ligase II—DNA ligase II was purified from bovine liver nuclei by measuring formation of the labeled 70-kDa enzyme-adenylate intermediate and joining of the oligo(dT)·poly(rA) substrate as described under "Materials and Methods." We chose to purify DNA ligase II from bovine liver since this tissue contains significantly higher levels of DNA ligase II activity compared with the thymus (15). Furthermore, during subcellular fractionation under isotonic conditions, DNA ligase II remains firmly associated with nuclei, whereas the majority of DNA ligase I leaks out of the nuclei and is found in the cytoplasmic/soluble fraction (15, 18). After the preparation of a nuclear extract, residual traces of DNA ligase I were removed by phosphocellulose chromatography. In contrast to experiments with rat liver (29), we were unable to detect 100-kDa DNA ligase III in either the cytoplasmic/soluble or nuclear fractions from bovine liver.

During the latter stages of the purification, DNA ligase II exhibited a tendency to precipitate. To improve the yield of DNA ligase II protein, a non-ionic detergent, Tween 20 (0.2%), was included in the buffers. In the last step of the purification, a single polypeptide of 70-kDa co-eluted with a similar sized polypeptide that formed a labeled enzyme-AMP complex (Fig. 1, *A* and *B*). We obtained approximately 20 µg of the 70-kDa polypeptide, which appeared to be greater than 90% homogeneous (Fig. 1*A*), from 2.5 kg of bovine liver.

In assays with the homopolymer substrates, DNA ligase II had a specific activity of 4 units/mg when assayed with the oligo(dT)·poly(rA) substrate compared with 2 units/mg when assayed with the oligo(dT)·poly(dA) substrate (data not shown). These values are similar to that obtained for homogeneous bovine DNA ligase I assayed with the oligo(dT)·poly(dA) substrate (2.5 units/mg) (24) but are significantly higher than the specific activity of apparently homogeneous DNA ligase II

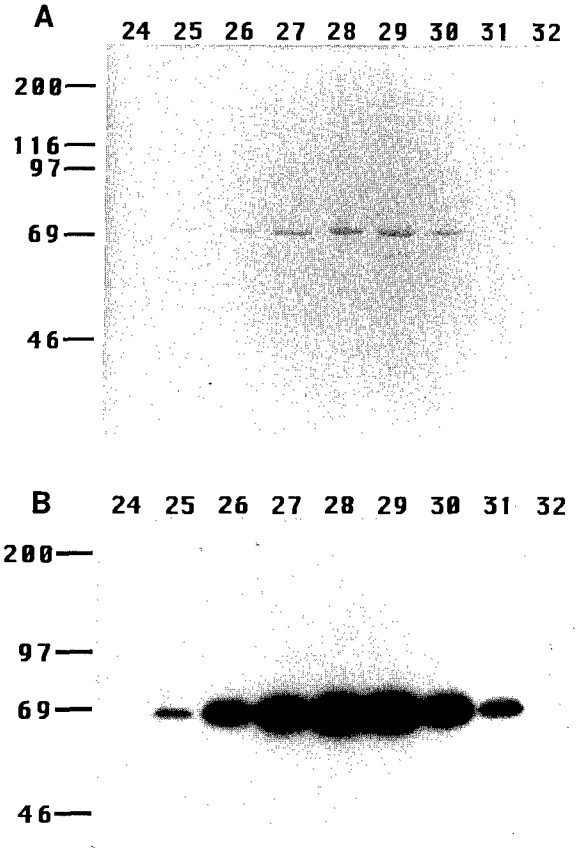

FIG. 1. **Purification of bovine DNA ligase II by FPLC Mono S chromatography.** Fractions containing DNA ligase II activity following FPLC Mono Q chromatography were applied to an FPLC Mono S column and gradient eluted as described under "Materials and Methods." Fractions containing protein, measured by absorbance at 280 nm, were analyzed. *A*, after aliquots (10 μl) of the indicated fractions were electrophoresed through a 10% denaturing polyacrylamide gel, proteins were stained with Coomassie Blue. The positions of size markers, myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 69 kDa; and ovalbumin, 46 kDa (Bio-Rad), are indicated on the *left*. *B*, aliquots (2 μl) of the indicated fractions were assayed for enzyme-AMP formation as described under "Materials and Methods." After separation through a 10% denaturing polyacrylamide gel, labeled polypeptides were detected by autoradiography. The positions of ^{14}C-labeled size markers, myosin, 200 kDa; phosphorylase *b*, 97 kD; bovine serum albumin, 69 kDa; and ovalbumin, 46 kDa (Amersham Corp.), are indicated on the *left*.

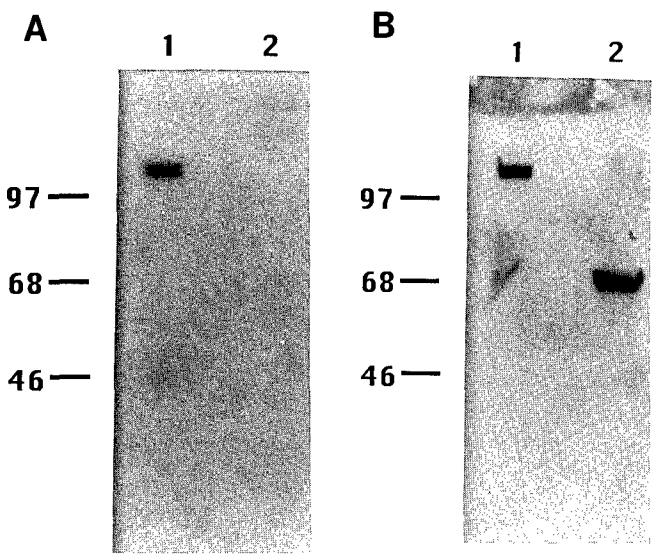

FIG. 2. **Bovine DNA ligase II cross-reacts with the antiserum raised against the conserved epitope present in several eukaryotic DNA ligases but not with an antiserum raised against bovine DNA ligase I.** Homogeneous human DNA ligase I and bovine DNA ligase II were electrophoresed through two identical 10% denaturing polyacrylamide gels and then transferred to nitrocellulose membranes. *A*, nitrocellulose membrane incubated with antiserum raised against homogeneous bovine DNA ligase I. *B*, nitrocellulose membrane incubated with antiserum raised against a peptide common to several eukaryotic DNA ligases. *Lane 1*, 50 ng of recombinant human DNA ligase I and *lane 2*, 200 ng of bovine DNA ligase II. Immune complexes were detected by enhanced chemiluminescence. The positions of size markers phosphorylase *b*, 97 kDa; bovine serum albumin, 68 kDa; and ovalbumin, 46 kDa (Amersham Corp.) are indicated on the *left*.

(0.001 unit/mg) isolated from calf thymus glands (1).

Although the three mammalian DNA ligases can be distinguished *in vitro* by their reactivity with the various homopolymer substrates, it is not apparent that these differences in substrate specificity are physiologically relevant. We have constructed a DNA substrate by hybridization of three appropriate oligonucleotides that contains a single defined nick with adjacent 5′-phosphate and 3′-hydroxyl termini within a linear duplex DNA molecule (24) assuming that this more closely resembles the *in vivo* substrate for DNA ligases. In assays with such a substrate, bovine DNA ligase II catalyzes phosphodiester bond formation, joining the end-labeled 16-mer to the adjacent 18-mer when these oligonucleotides are hybridized to a complementary 34-mer (data not shown).

Bovine DNA Ligase II Cross-reacts with the Antiserum Raised against the Conserved COOH-terminal Peptide Sequence Present in Mammalian DNA Ligase I, Yeast DNA Ligases, and Vaccinia DNA Ligase—During purification, proteolytic cleavage of mammalian DNA ligase I by endogenous proteases can produce catalytically active fragments (25). To demonstrate that the 70-kDa polypeptide was not an active proteolytic fragment of DNA ligase I, we performed immunoblotting experiments with a polyclonal antiserum raised against homogeneous bovine DNA ligase I (25). As expected this antiserum recognized purified recombinant human DNA ligase I (Fig. 2*A*) and partially purified bovine DNA ligase I (data not shown) but not bovine DNA ligase II (Fig. 2*A*).

Mammalian DNA ligase I, vaccinia DNA ligase, Cdc9 DNA ligase, and Cdc17 DNA ligase all contain a conserved 16-amino acid sequence close to their COOH termini (2, 6). In contrast to a previous report (1), we find that the antiserum raised against this conserved COOH-terminal peptide sequence (25) cross-reacts with bovine DNA ligase II (Fig. 2*B*). Since the specific activity of this DNA ligase II preparation is 3000-fold higher than that of apparently homogeneous DNA ligase II from calf thymus (1), it appears likely that DNA ligase II was a minor component of the calf thymus preparation and was not present in sufficient quantity for detection by immunoblotting.

Inhibition of DNA Ligase II-Adenylate Formation by Pyridoxal Phosphate—In the first step of the ligation reaction, DNA ligase reacts with either ATP (bacteriophage, viral, and eukaryotic enzymes) or NAD^+ (bacterial enzymes) to form a covalent enzyme-adenylate intermediate. For *Escherichia coli* DNA ligase, bacteriophage T4 DNA ligase (30), and mammalian DNA ligase I (23), it has been demonstrated that the AMP residue is linked to a lysine residue via a phosphoramidite bond. Adenylylated DNA ligase II is sensitive to treatment with acidic hydroxylamine (data not shown), which cleaves phosphoramidite bonds (30). Since internal histidine residues also possess amino groups that can potentially form phosphoramidite bonds, we cannot conclude that the AMP group is linked via a lysine residue. To provide further evidence for the direct involvement

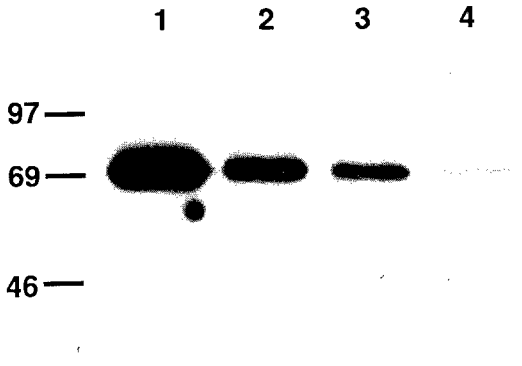

FIG. 3. **Inhibition of enzyme-adenylate formation by pyridoxal phosphate.** DNA ligase II (40 ng) incubated with; *lane 1*, no addition, *lane 2*, 50 μM PLP; *lane 3*, 100 μM PLP and *lane 4*, 200 μM PLP as described under "Materials and Methods." After 5 min, 2 μCi of [α^{32}P]ATP was added, and the incubation was continued for 15 s. After separation by SDS-PAGE, labeled enzyme-adenylate complexes were detected by autoradiography. The positions of ^{14}C-labeled size markers, phosphorylase *b*, 97 kDa; bovine serum albumin, 69 kDa; and ovalbumin, 46 kDa (Amersham Corp.) are indicated on the *left*.

of a lysine residue in the adenylylation reaction of mammalian DNA ligase II, the enzyme was incubated with pyridoxal phosphate, which specifically interacts with reactive, nucleophilic lysine residues (23, 31), prior to the addition of labeled ATP. Formation of DNA ligase II-adenylate was inhibited 75% by 50 μM PLP, 84% by 100 μM PLP, and 94% by 200 μM PLP (Fig. 3). A similar degree of inhibition by PLP was observed with mammalian DNA ligase I (23).

Isolation and Amino Acid Sequence of a DNA Ligase II Active Site Peptide—To identify the active site lysine residue of DNA ligase II, homogeneous bovine DNA ligase II (290 pmol) was adenylylated with [^{3}H]ATP and then digested with a protease, endoproteinase Lys-C, that specifically cleaves the peptide bond on the COOH-terminal side of lysine residues. Adenylylated lysine residues are not recognized as a substrate by lysine-specific proteases (23, 32). The resultant peptides were separated by reverse phase HPLC as described under "Materials and Methods." The majority of the radioactivity, corresponding to about 60 pmol of AMP, eluted from the column in a single fraction, which was coincident with a major peak of absorbance at 260 nm and a smaller peak of absorbance at 280 nm (data not shown). The A_{260}/A_{280} ratio of this peak was 1.5, which is consistent with the presence of adenylyl residues.

Amino acid sequence analysis of this fraction revealed the presence of a single major peptide with the sequence Cys-Pro-Asn-Gly-Met-Phe-Ser-Glu-Ile-Lys-Tyr-Asp-Gly-Glu-Arg-Val-Gln-Val-His. The predicted, internal lysine residue was detected at position 10 of this 19 amino acid sequence. Since we were unable to detect a signal in the 20th and subsequent cycles of Edman degradation, we do not know whether the peptide terminated with a lysine at position 20 or continued. Edman degradation chemistry involves highly acidic conditions that preclude identification of a lysine-AMP residue. However, the presence of an internal protease-resistant lysine residue implies that this residue was the site of adenylylation. Inhibition of cleavage by lysine-specific proteases at lysine-AMP residues has been observed for the adenylylated forms of yeast tRNA ligase (32) and bovine DNA ligase I (23). Since the amino acid sequences of 19 Lys-C peptides from a similar quantity of non-adenylylated DNA ligase II digested under identical conditions did not contain internal lysine residues (see later), the lysine residue detected in the 10th Edman degradation cycle is unlikely to be a consequence of incomplete digestion. The yield

```
                                    *
Human DNA ligase I   556  KRFEEAAFTCEYKYDGQRAQIHALEGGEVK 585
                               + ++++ + + +
Bovine DNA ligase II          CPNGMFSEIKYDGERVQVH
                                + +++ + ++++++++++
Vacc. DNA ligase     218  FKKFPSGMFAEVKYDGERVQVHKNNNEFAF 248
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FIG. 4. **Alignment of active site sequences from mammalian DNA ligase I and vaccinia DNA ligase with the active site region of bovine DNA ligase II.** Alignment of the adenylylated DNA ligase II peptide with the apparently related sequences in the human DNA ligase I gene (2) and the vaccinia virus DNA ligase gene (6) is shown. Amino acids are numbered from the amino-termini of the full-length polypeptides. An adenylylated tryptic peptide isolated from bovine DNA ligase I had the amino acid sequence, FEEAAFT-EYKYDGQR, which corresponds exactly with residues 558–573 of translated human DNA ligase I cDNA (23). The 6-amino acid active site motif that is conserved in all DNA ligases is indicated in *boldface*. The *asterisk* indicates the postulated or known site of adenylylation. Amino acids conserved between the DNA ligases are marked with a *cross*.

of the phenylthiohydantoin-derived amino acids during Edman degradation indicated that the fraction contained about 70 pmol of peptide, consistent with a 1:1 ratio of AMP and peptide molecules. Furthermore, the A_{260}/A_{280} ratio of 1.5 is close to the predicted value for a peptide containing one tyrosine and one AMP residue.

The lysine residue in the DNA ligase II active site peptide was part of a 6-amino acid sequence which is homologous to the active site motif, Lys-Tyr/Ala-Asp-Gly-Xaa-Arg, found in all ATP-dependent DNA ligases (23) (Fig. 4). A comparison of the 19-amino acid adenylylated Lys-C peptide from bovine DNA ligase II with the corresponding sequence encoded by human DNA ligase I cDNA (2, 23) revealed clear differences between these enzymes in the regions flanking the active site motif (Fig. 4). The alterations in amino acid sequence (two changes in 4 residues) in the region COOH-terminal to the active site motif could be explained as minor changes in functionally homologous gene products from different species. Since the amino acid sequence of an adenylylated tryptic peptide from bovine DNA ligase I corresponds exactly to residues 558–573 encoded by human DNA ligase I cDNA, the changes in amino acid sequence within this region (eight differences in a 9-amino acid sequence) unequivocally demonstrate that DNA ligase II is not a proteolytic fragment of DNA ligase I and provide compelling evidence that these enzymes are encoded by distinct genes.

The active site region of DNA ligase II is most closely related to the active site region of the DNA ligase encoded by vaccinia virus, with 15 identical residues out of 19 (Fig. 4). During the sequencing of this peptide, we did not detect the COOH-terminal lysine residue anticipated from a Lys-C digestion. Progressive reduction in signal toward the COOH-terminal residue of a peptide is a common feature of amino acid sequencing. The homology between the DNA ligase II active site peptide and the active site region of vaccinia DNA ligase suggests that the lysine residues in the vaccinia DNA ligase sequence that flank the DNA ligase II peptide may be conserved in bovine DNA ligase II (Fig. 4).

The Catalytic Domain of Mammalian DNA Ligase II Is More Closely Related to Vaccinia DNA Ligase than to Mammalian DNA Ligase I—A surprising feature of the adenylylated DNA ligase II peptide was the high degree of homology with the active site region of vaccinia DNA ligase. To investigate the extent of the relationship between mammalian DNA ligase II and vaccinia DNA ligase, we obtained more amino acid sequence information from bovine DNA ligase II.

Attempts to determine the NH_2-terminal amino acid sequence of the 70-kDa polypeptide were unsuccessful. Since we were able to obtain amino acid sequences from the same preparation of DNA ligase II after proteolytic digestion (see below), we concluded that, as is the case with the majority of soluble proteins in mammalian cell extracts (33), the NH_2 terminus of

```
Lig I     KYDGQRAQIHALEGGEVKIFSRNQEDNTGKYPDIISRIPKIKLPSVTSFILDT
          ++++ + + +   +     +++                   +  +   +  ++
Lig II    KYDGERVQVE  KGDHFSYFSRSLK           KDFIPIAFPGGHSMILDS
          ++++++++++       +  +++  +          +  ++ ++    +  +++
Vacc      KYDGERVQVHK NNNEFAFFSRNMKPVLSHKVDYLKEYIPKAFKKATSIVLDS

Lig I     EAVAWDREKK QIQPFQVLTTRKRKEVDASEIQVQVCLYAFDLIYLNGESLVR
          +                                 +  + +  +
Lig II    EVLL                    AAFQDANVCLFVFDCIYFNDVSLMDRPL-E
          +  +                           + +++++++ ++       + ++ +
Vacc      EIVLVDEHNVPLPFGSLGIHKKKEYKNSNMCLFVFDCLYFDGFDMTDIPLYE

Lig I     EPLSRRRQLLRENFVETEGEFVFATSLDTKDIEQIAEFLEQSVKDSCEGLMVK
                ++  + ++      +
Lig II            LLHDNMVEIPNAIMFSEMK
                   + + ++++++ + +++
Vacc           RRSFLKDVMVEIPNRIVFSELTNISNESQLTDVLDDALTRKLEGLVLK

Lig I     TLDVDATYEIAKRSHNWLKLKKDYLD G VGDTLDLVVIGAYLGRGKRAGRYG
                +++  +    +++   +++  +   ++ ++++ ++  + +    +
Lig II          GTYEPGK  RHWLK   DYLNEGAMADTADLVVLGAFYGQGSKGGMMS
                + +++++  + +++   ++++++ +++ ++++++++ ++ + +++ +
Vacc      DIN   GVYEPGK  RRWLKIKRDYLNEGSMADSADLVVLGAYYGKGARGGIMA

Lig I     GFLLASYDEDSEELQAICKLGTGFSDEELEEHHQSLKALVLPSPRPYVRIDGA
          ++   ++  +          +   +  +  +        +
Lig II    IFLMGCYDPSSQKWCTVTKCAGGHDDATLARLQG-LDMVK         IPNWLK
           +++++++  + ++ +++++ + +++ ++  ++  + +++         ++ ++
Vacc      VFLMGCYDDESGKWKTVTKCSG HDDNTLRVLQDQLTMVKINKDPKKIPEWL

Lig I     VIPDHWLDPSAVWAVKCADLSLSPIYPAARGLVDSDKGISLRFPRFIRVREDK
                     +   + +                      +++ ++++
Lig II         IYYPDFIVPDPK AAVW-ITGA-FS SEAHTADGISIRFPR-T-I
               ++ +++ + +++    + + ++ +      +++ ++++++++ + +
Vacc      VVNKIYIPDFVVEDPKQSQIWEISGAEFTSSKSHTANGISIRFPRFTRIREDK

Lig I     QPEQATTSAQVACLYRKQSQIQNQQGEDSGSDPEDTY
Lig II
Vacc      TWKESTHLNDLVNLTKS
```

FIG. 5. **Amino acid sequence homology between bovine DNA ligase II peptide sequences and the catalytic domains of human DNA ligase I and vaccinia DNA ligase.** The predicted amino acid sequences from the putative active site lysine residues to the C termini of human DNA ligase I (568–919) and vaccinia DNA ligase (231–552) have been aligned. Amino acids are numbered from the amino termini of the full-length polypeptides. Gaps have been introduced for maximum alignment. The alignment of bovine DNA ligase II peptides with apparently related sequences in human DNA ligase I and vaccinia DNA ligase is shown. A hyphen indicates a position within a peptide where it was not possible to unambiguously assign an amino acid. Amino acids conserved between the DNA ligases are marked with a *cross*.

the 70-kDa polypeptide was blocked to Edman degradation. After digestion of DNA ligase II with endopeptidase Lys-C and separation of the resultant peptides by reverse phase HPLC, amino acid sequences were obtained from 19 different peptides, in addition to the active site peptide described previously. A comparison of these sequences with the 63-kDa vaccinia DNA ligase (Fig. 5) revealed that 14 of the peptide sequences were homologous with this enzyme (6). The peptides were aligned within the region from the active site to the COOH terminus. The degree of identity ranged from 38 to 86% and generally increased toward the COOH terminus. The DNA ligase genes of two other poxviruses, variola and Shope fibroma virus, have also been sequenced (34, 35). The predicted amino acid sequences of vaccinia and variola DNA ligases exhibit 98% identity whereas they share only 45% identity with the Shope fibroma DNA ligase. The DNA ligase II peptides are more highly related to vaccinia and variola DNA ligases (65% overall identity) than Shope fibroma DNA ligase (57% overall identity).

One of the DNA ligase II peptides, Ser-Glu-Ala-His-Thr-Ala-Asp-Gly-Ile-Ser-Ile-Arg-Phe-Pro-Arg-Xaa-Thr-Xaa-Ile, is partly homologous with the conserved peptide sequence present in eukaryotic and vaccinia DNA ligases (2, 6). Presumably this peptide forms part of the DNA ligase II epitope that was recognized by the antiserum raised against the conserved peptide sequence in the immunoblotting experiment (Fig. 2). The other bovine DNA ligase II peptides also share homology with the catalytic domain of human DNA ligase I (Fig. 5). Within this region, however, DNA ligase II is more similar to vaccinia DNA ligase, exhibiting 65% identity compared to 31% with human DNA ligase I. Thus, the catalytic domain of mammalian DNA ligase I is more closely related to the DNA ligases encoded by the *S. cerevisiae CDC9* gene and the *S. pombe Cdc17*$^+$ gene (2) than to mammalian DNA ligase II.

DISCUSSION

Despite immunological and biochemical evidence indicating that mammalian DNA ligases I and II are encoded by separate genes (1, 14–16), this hypothesis has remained controversial (36, 37). In this study, we have determined the amino acid sequence of an adenylylated peptide isolated from homogeneous bovine DNA ligase II. This peptide, Cys-Pro-Asn-Gly-Met-Phe-Ser-Glu-Ile-Lys-Tyr-Asp-Gly-Glu-Arg-Val-Gln-Val-His, contains a sequence that is homologous with the active site motif Lys-Tyr/Ala-Asp-Gly-Xaa-Arg found in ATP-dependent DNA ligases, including mammalian DNA ligase I (23). The clear, unambiguous differences between DNA ligase I and DNA ligase II in the region adjacent to the active site motif provides compelling evidence that these enzymes are encoded by separate genes.

We believe that the lysine residue at position 10 within the DNA ligase II peptide sequence is the site of adenylylation for the following reasons; (i) the enzyme-adenylate complex was sensitive to acidic hydroxylamine, indicating the presence of a phosphoramidite bond (30); (ii) enzyme-adenylate formation was inhibited by PLP, a reagent specific for nucleophilic lysine residues (23, 31); (iii) the AMP and peptide moieties were present in a 1:1 molar ratio; (iv) the amino acid sequence of the Lys-C adenylylated peptide contains an internal Lys-C-resistant lysine residue that forms part of an active site motif found in all ATP-dependent DNA ligases. The essential role of the lysine residue within the active site motif of human DNA ligase I and vaccinia DNA ligase has been verified by site specific mutagenesis (38, 39). A reactive lysine residue in a shortened version of the motif, Lys-Xaa-Asp-Gly, has also been identified as the active site of RNA ligases (32, 40) and of GTP-dependent mRNA capping enzymes (39, 41, 42).

The DNA ligase II active site peptide exhibits most homology (15 identical residues out of 19) with the active site region of vaccinia DNA ligase. The homologous sequence in vaccinia DNA ligase is flanked by lysine residues. If we assume that these residues are situated in the same positions within bovine DNA ligase II, the predicted peptides generated by trypsin, Lys-C and V8 protease are consistent with the peptide mapping data obtained by Roberts *et al.* (43). A comparison of the amino acid sequences of another 19 Lys-C DNA ligase II peptides with vaccinia DNA ligase revealed that 14 of these peptides were homologous with the region from the active site to the COOH terminus of this enzyme. The overall degree of identity between the homologous DNA ligase II peptides and the putative catalytic domain of vaccinia DNA ligase was 65%, significantly higher than the 31% identity observed in a similar comparison with the catalytic domain of human DNA ligase I. From this analysis, it is apparent that DNA ligase II is more closely related to vaccinia DNA ligase whereas the replicative DNA ligases, mammalian DNA ligase I, *S. cerevisiae CDC9* and *S. pombe Cdc17*$^+$, are more closely related to each other (2) than to DNA ligase II.

Based on similarities in substrate specificity and the generation of similar adenylylated peptides, it has been suggested that DNA ligase II and III may be generated from a common precursor polypeptide by differential processing or from the same gene by alternative splicing (43, 44). Since DNA ligase II is homologous with other eukaryotic DNA ligases, it is probable that this enzyme has the same domain structure as all other

ATP-dependent DNA ligases, consisting of a catalytic COOH-terminal domain and a unique NH_2-terminal domain (23, 25). Therefore, DNA ligase II would have to be generated from the 100-kDa DNA ligase III by removal of an amino-terminal fragment. However, the NH_2 terminus of the 70-kDa DNA ligase II polypeptide was blocked to Edman degradation, implying that it retains the *N*-acetylated amino-terminal residue of the primary translation product (33). Furthermore, a polyclonal antibody raised against the 68-kDa bovine DNA ligase II did not recognize higher molecular weight polypeptides (45) and a proteolytic fragment similar in size to DNA ligase II was not generated by proteolytic cleavage of DNA ligase III (1). Thus, it appears that DNA ligases II and III are not derived from a common polypeptide but may be generated by alternative splicing or encoded by separate genes.

The high degree of identity between the catalytic domains of mammalian DNA ligase II and vaccinia DNA ligase, and the similar sizes of these enzymes (6, 11, 12) suggests that this cytoplasmic virus recruited the host DNA ligase II gene. We have not been able to unambiguously align the remaining five DNA ligase II peptide sequences with homologous regions of vaccinia DNA ligase. This absence of homology, in particular with the amino-terminal region of vaccinia DNA ligase, may reflect either the lack of selective pressure on a region of the protein that is not required for catalytic function or, alternatively, selective pressure to evolve a DNA ligase that specifically interacts with vaccinia proteins. A similar lack of homology has been observed in a comparison of the amino-terminal regions of mammalian DNA ligase I, *S. cerevisiae* Cdc9 and *S. pombe* Cdc17 DNA ligase (2).

Vaccinia DNA ligase, which has been detected in discrete cytoplasmic factories where viral DNA synthesis occurs, is not essential for virus DNA replication (11, 12). It is possible that, in the absence of DNA ligase, vaccinia can replicate by a mechanism that does not require joining of Okazaki fragments. Alternatively, one of the host DNA ligases may be able to substitute for the viral enzyme. Expression of the vaccinia DNA ligase gene can rescue the conditional lethal phenotype of a *S. cerevisiae cdc9* DNA ligase mutant (12). This type of complementation does not necessarily implicate this enzyme in DNA replication but, more likely, reflects the uncoupling of replication fork progression from Okazaki fragment joining with the subsequent joining of strand breaks in the lagging strand by any available DNA ligase. The mutant vaccinia virus lacking the DNA ligase gene was more sensitive to treatment with DNA damaging agents than the wild type virus, implying that the viral enzyme may be involved in DNA repair (12). A similar cellular function has been predicted for DNA ligase II (19, 20).

In summary, amino acid sequence analysis of mammalian DNA ligase II demonstrates that this enzyme is encoded by a gene distinct from the one encoding DNA ligase I (2, 3). However, the homology between the catalytic domains of DNA ligases I and II indicates that the genes encoding these enzymes probably evolved from a common eukaryotic ancestral gene. Mammalian DNA ligase II is more highly related to vaccinia DNA ligase, suggesting that the recruitment of this host gene by vaccinia occurred much more recently than the divergence of the genes encoding DNA ligase I and DNA ligase II. Based on the phenotypic properties of vaccinia DNA ligase mutants (11, 12) and previous biochemical studies on DNA ligase II (19, 20), it is our working hypothesis that DNA ligase II functions in certain housekeeping DNA repair pathways. The availability of partial amino acid sequence from mammalian DNA ligase II should facilitate the isolation of the gene encoding this enzyme and further investigation of it's relationship with DNA ligase III and it's function(s) in DNA metabolism.

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Purification and Characterization of DNA Ligase III from Bovine Testes

HOMOLOGY WITH DNA LIGASE II AND VACCINIA DNA LIGASE*

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Mammalian cell nuclei contain three biochemically distinct DNA ligases. In the present study we have found high levels of DNA ligase I and DNA ligase III activity in bovine testes and have purified DNA ligase III to near homogeneity. The high level of DNA ligase III suggests a role for this enzyme in meiotic recombination. In assays measuring the fidelity of DNA joining, we detected no significant differences between DNA ligases II and III, whereas DNA ligase I was clearly a more faithful enzyme and was particularly sensitive to 3′ mismatches. Amino acid sequences of peptides derived from DNA ligase III demonstrated that this enzyme, like DNA ligase II, is highly homologous with vaccinia DNA ligase. The absence of unambiguous differences between homologous peptides from DNA ligases II and III (10 pairs of peptides, 136 identical amino acids) indicates that these enzymes are either derived from a common precursor polypeptide or are encoded from the same gene by alternative splicing. Based on similarities in amino acid sequence and biochemical properties, we suggest that DNA ligases II and III, *Drosophila* DNA ligase II, and the DNA ligases encoded by the pox viruses constitute a distinct family of DNA ligases that perform specific roles in DNA repair and genetic recombination.

DNA joining is required to link together Okazaki fragments during lagging strand DNA synthesis and to seal DNA strand breaks produced either by the direct action of a damaging agent or by DNA repair enzymes removing DNA lesions. In addition, DNA ligation is necessary to complete exchange events between homologous duplex DNA molecules. Prokaryotes contain a single species of DNA ligase that presumably functions in each of the above DNA metabolic pathways (1). In contrast, three biochemically distinct DNA ligases have been identified in extracts from mammalian cells (2).

In *in vitro* assays DNA ligase I appears to be the enzyme that joins Okazaki fragments during DNA replication (3–5). The abnormal pattern of DNA replication intermediates detected in experiments with the human cell line 46BR and its derivatives, which contain mutated DNA ligase I alleles, are consistent with an *in vivo* defect in Okazaki fragment joining (6–9). Furthermore, the sensitivity of these cell lines to DNA damaging agents suggests that DNA ligase I may also be involved in certain DNA repair pathways (6, 10–12).

The high levels of DNA ligase I activity in the thymus of young animals facilitated the purification of this enzyme to homogeneity from calf thymus glands (13, 14). Two minor DNA ligase activities, designated as DNA ligase II and DNA ligase III, have also been identified in calf thymus extracts (2, 15, 16). The 70-kDa DNA ligase II, which is the major DNA joining activity in the normal liver (17), is not recognized by a polyclonal antiserum specific for DNA ligase I (2, 15, 18, 19). Recent amino acid sequencing studies with homogeneous bovine DNA ligase II confirmed that this enzyme is not a proteolytic fragment of DNA ligase I and revealed that the enzyme is highly homologous with the DNA ligase encoded by vaccinia virus (18). It has been reported that the level of DNA ligase II activity is induced by DNA damage, suggesting that it may play a role in DNA repair (20, 21).

The 100-kDa DNA ligase III is also not recognized by the DNA ligase I-specific antiserum (2). However, the relationship between DNA ligase II and DNA ligase III is less clearly defined. Based on differences in the physical, catalytic, and chromatographic properties of these enzymes, it was concluded that they are probably encoded by separate genes (2). In contrast, a recent comparison of DNA ligase adenylation sites by peptide mapping demonstrated significant similarities between the active sites of these enzymes, suggesting that they may be related by alternative splicing (22). The association of DNA ligase III with a calf thymus recombination complex (23) and with a human DNA repair protein, XRCC1 (24), is consistent with this enzyme joining DNA strand breaks to complete recombination and repair events.

In this report we describe the purification of DNA ligase III to near homogeneity from bovine testes. Amino acid sequencing studies have revealed a high degree of homology between DNA ligase III and vaccinia DNA ligase. Furthermore, many of the DNA ligase III peptides were identical with peptides isolated from bovine DNA ligase II. The absence of unambiguous differences between homologous DNA ligase II and III peptides indicates that these enzymes are either derived from a common precursor polypeptide or are encoded from the same gene by alternative splicing.

MATERIALS AND METHODS

Purification of Recombinant Human DNA Ligase I—Human DNA ligase I cDNA was subcloned into a baculovirus expression vector, pVL1392 (PharMingen). The details of the purification of recombinant human DNA ligase I from baculoviral-infected insect cells will be described elsewhere. In assays with the oligo(dT)/poly(dA) substrate, ho-

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mogeneous 125-kDa DNA ligase I had a specific activity of 2.5 units/mg.

Partial Purification of DNA Ligase I and DNA Ligase III from Whole Cell Extracts of Bovine Testes—Testes from newly slaughtered bulls were kept on ice and processed within 3 h. A cell-free extract was prepared from 250 g of bovine testes by homogenization and then fractionated by phosphocellulose chromatography, ammonium sulfate precipitation, and gel filtration as described by Tomkinson *et al.* (2). Protein concentrations were measured by the method of Bradford (25). Fractions eluting from the gel filtration column were assayed for DNA joining activity with both the oligo(dT)/poly(dA) and oligo(dT)/poly(rA) substrates and for enzyme-adenylate formation. Fractions containing both DNA ligase I and DNA ligase III activity (2, 8) were pooled and fractionated by hydroxylapatite chromatography (26). Consistent with previous results, the majority of DNA ligase I activity was eluted by 150 mM potassium phosphate. DNA ligase I was further purified by native DNA cellulose chromatography and FPLC[1] Mono Q chromatography as described (14) and was approximately 30% homogeneous.

DNA ligase III activity, which was eluted by 400 mM potassium phosphate, was dialyzed against 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 10% glycerol (buffer A) and applied to a native DNA cellulose column. Bound proteins were eluted stepwise with 0.2 and 0.5 M NaCl in buffer A. Active fractions, which eluted with 0.5 M NaCl, were dialyzed against buffer A and then applied to an FPLC Mono Q 5/5 column. Bound proteins were eluted with a 20-ml linear gradient from 0.05–0.75 M NaCl in buffer A. DNA ligase III activity eluted at 250 mM NaCl. A 100-kDa polypeptide detected by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis co-eluted with DNA ligase activity. Assuming that this polypeptide was responsible for the labeled 100-kDa enzyme-adenylate, this preparation of DNA ligase III was approximately 30% homogeneous.

For amino acid sequencing studies, the peak fractions of DNA ligase III from the FPLC Mono Q column (2 ml, 25 μg) were pooled and concentrated by ultrafiltration using a Centricon-10 apparatus (Amicon) that had been pretreated with 2% Triton X-100. Polypeptides (400 μl) were separated by electrophoresis through a preparative 10% SDS-polyacrylamide gel and then transferred to a polyvinylidene membrane (Bio-Rad). After staining with Ponceau S, the strip of membrane containing the 100-kDa DNA ligase III was excised and washed with distilled H_20. After digestion *in situ* with trypsin, the resultant peptides were separated by reverse phase HPLC (27).

Purification of DNA Ligase III from Testis Nuclei—Three testes (0.75 kg) were sliced into 1-inch cubes, resuspended in 1 liter of buffer B (50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.8 mM phenylmethylsulfonyl fluoride, 0.2 mM Pefabloc (Boehringer Mannheim), 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 3.5 μg/ml TPCK, 25 μg/ml TLCK, and 1 mM benzamidine) and homogenized in a Waring blender. The homogenate was filtered through cheesecloth with buffer B added periodically to maintain a volume of about 1.5 liters. Nuclei were collected by centrifugation at 2500 × *g* for 30 min and washed three times with buffer B.

The crude nuclei (40 g) were resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 750 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM Pefabloc, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 μg/ml chymostatin, 3.5 μg/ml TPCK, 25 μg/ml TLCK, and 1 mM benzamidine (buffer C) and then lysed by Dounce homogenization. After the addition of 40% polyethylene glycol 8000 to a final concentration of 5%, the suspension was stirred for 15 min and then centrifuged at 10,000 rpm for 10 min in a GSA rotor (Sorvall). The clarified nuclear extract (160 ml, 247 mg) was adjusted to 1 mM potassium phosphate and then loaded onto a 35-ml hydroxylapatite column that had been equilibrated with buffer C containing 1 mM potassium phosphate. Proteins were eluted stepwise with 50, 150, and 400 mM potassium phosphate (pH 7.5) buffers containing 1.0 mM DTT and protease inhibitors as described in buffer C. DNA ligase III activity, which was eluted in the 400 mM fraction (30 ml, 90 mg), was diluted 1 in 4 with 67 mM NaCl, 1.33 mM EGTA, and 1.33 mM DTT and loaded onto a 6.5-ml P11 phosphocellulose column that had been equilibrated with buffer D (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, and protease inhibitors as described in buffer C). Bound proteins were eluted stepwise with 100, 250, and 450 mM NaCl sequentially in buffer D. DNA ligase III activity was detected in the 450 mM eluate (12 ml, 22 mg). The samples were then diluted 1 in 6 with buffer D without NaCl to adjust the NaCl to 75 mM and loaded onto a 5-ml native DNA-cellulose column equilibrated with buffer D. Bound proteins were eluted stepwise with buffer D containing 200 mM and 500 mM NaCl. DNA ligase III activity (9 ml, 5 mg), which was eluted in the 500 mM NaCl buffer, was loaded onto an AcA34 gel filtration column (2.6 × 98 cm) that had been equilibrated with buffer D containing 1 M NaCl. Active fractions were pooled, dialyzed against buffer D, and then loaded onto an FPLC Mono Q HR 5/5 column. Bound proteins were eluted with a 20 ml of linear gradient from 50 to 750 mM NaCl in buffer D. DNA ligase III (0.7 ml, 35 μg), which eluted at about 250 mM NaCl, was stored in aliquots at −80 °C. Under these storage conditions, the enzyme was stable for at least 6 months.

Preparation of Substrates for DNA Joining Assays—Polynucleotides dA, rA, and dT were purchased from Pharmacia Biotech Inc. $Oligo(dT)_{30}$ was synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer. Labeled homopolymer substrates were prepared as described previously (28). Labeled polynucleotide substrates containing a single, defined nick were prepared by annealing three complementary oligonucleotides as described previously (28).

DNA Ligase Assays—Phosphodiester bond formation was assayed as described previously (28). One unit of DNA ligase activity catalyzes the conversion of 1 nmol of terminal phosphate residues to a phosphatase-resistant form in 15 min at 20 °C.

Analysis of Ligation Products—Aliquots (10 μl) from DNA ligase assays were added to 10 μl of formamide dye and heated for 2 min at 90 °C. Samples (2.5 μl) were then loaded onto a denaturing 10% polyacrylamide gel. After electrophoresis, the gels were dried and oligonucleotides were visualized by autoradiography. Formation of ligated products was quantitated by phosphorimage analysis (Molecular Dynamics).

Formation of DNA Ligase-Adenylate—The adenylation reactions (12 μl) were routinely carried out in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 50 μg/ml bovine serum albumin, 0.5–3.0 μCi [α-^{32}P] ATP (3000 Ci/mmol, Amersham Corp.) and the enzyme fraction (29). After incubation at room temperature for 15 min, reactions were stopped by the addition of an equal volume of 2 × SDS sample buffer. Samples were heated at 90 °C for 5 min and polypeptides were separated by electrophoresis through an 8% SDS-polyacrylamide gel (30). Gels were fixed in 10% acetic acid and dried. Adenylylated polypeptides were detected by autoradiography.

Immunoblotting—Proteins were separated by denaturing polyacrylamide gel electrophoresis (30) and transferred to nitrocellulose membranes. After incubation with either antiserum raised against homogeneous bovine DNA ligase I (14) or antiserum raised against the conserved COOH-terminal peptide of eukaryotic DNA ligases (14), antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham).

Proteolytic Digestion and Amino Acid Sequencing of Bovine DNA Ligase III Peptides—Peptide sequences were obtained from both the partially purified and the near homogeneous preparations of DNA ligase III. DNA ligase III peptides from the partially purified preparation were isolated as described above. Near homogeneous DNA ligase III (10–15 μg) was applied to a hydrophobic sequencing column (Hewlett-Packard) according to the manufacturer's instructions. After *in situ* digestion with endoproteinase Lys-C (Wako), peptides were separated by reverse phase HPLC using a Spheri 5 ODS (Brownlee) column (27). The amino acid sequences of peptides were determined by automated Edman degradations performed on the ABI477A protein sequencer with the 120A phenylthiohydantoin analyzer.

RESULTS

Partial Purification of DNA Ligase I and DNA Ligase III from Whole Cell Extracts of Bovine Testes—Three biochemically distinct DNA ligase activities have been identified in whole cell extracts from calf thymus glands (2). Since the high levels of DNA ligase I activity in this tissue hinders the purification of DNA ligases II and III, we have examined the relative levels of the DNA ligases in other bovine tissues. Recently, we have described the purification of DNA ligase II to homogeneity from liver nuclei (18). We did not detect DNA ligase III in significant quantities in liver extracts, and, therefore, we investigated the levels of DNA ligase III in testes. In order to compare the relative levels of DNA ligase III in the thymus and testes, we employed the same fractionation procedure used to purify DNA ligase III from calf thymus glands (2). After separation by gel filtration, a major peak of high molecular weight

[1] The abbreviations used are: FPLC, fast protein liquid chromatography; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

DNA joining activity containing both DNA ligase I and DNA ligase III was detected in assays with both the oligo(dT)/poly(dA) and oligo(dT)/poly(rA) substrates. Since DNA ligase I is not active with the oligo(dT)/poly(rA) substrate (2), the joining activity measured with this substrate reflects DNA ligase III activity. The specific activity of DNA ligase III was 4–5-fold higher in fractions from the testes compared with similar fractions from calf thymus glands (2), demonstrating that the testes contain significantly higher levels of DNA ligase III.

To determine the relative contribution of DNA ligase I and DNA ligase III to the high molecular weight DNA joining activity, the pooled fractions from the gel filtration column were fractionated by hydroxylapatite chromatography. Consistent with previous observations (14, 26), the majority of DNA ligase I was eluted with 150 mM potassium phosphate, whereas DNA ligase III was eluted with 400 mM potassium phosphate. The 400 mM eluate contained approximately 2-fold more DNA joining activity, measured with the oligo(dT)/poly(dA) substrate, than the 150 mM eluate. Thus, it appears that DNA ligase III is a major DNA joining activity in the testes.

Purification of DNA Ligase III from Testis Nuclei—Although DNA ligase I is a nuclear enzyme (31), this enzyme rapidly leaks out of nuclei during subcellular fractionation and is mainly found in the cytoplasmic/soluble fraction (19). In contrast, DNA ligase II remains firmly associated with nuclei isolated under isotonic conditions (18, 19, 32). The majority of DNA ligase III activity also remains associated with similarly prepared nuclei from bovine testes. In assays measuring enzyme-adenylate formation, the major labeled product in testis nuclear extracts corresponds to the 100-kDa DNA ligase III, with the 125-kDa DNA ligase I contributing about 5% and the 70-kDa DNA ligase II less than 1% (data not shown). Similarly prepared nuclear extracts from bovine liver also contain low levels of DNA ligase I, but in this tissue, the 70-kDa DNA ligase II is the predominant enzyme (18).

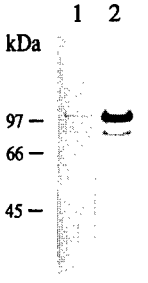

FIG. 1. **Analysis of purified bovine DNA ligase III by SDS-polyacrylamide gel electrophoresis.** Polypeptides were separated by electrophoresis through an 8% SDS-polyacrylamide gel. *Lane 1*, the peak fraction of DNA ligase III (400 ng) from testis nuclei after FPLC Mono S chromatography. Proteins were detected by staining with Coomassie Brilliant Blue; *lane 2*, 50 ng of the same fraction was assayed for enzyme-adenylate formation as described under "Materials and Methods." The positions of size markers, 97-kDa phosphorylase *b*, 66-kDa bovine serum albumin, and 45-kDa ovalbumin (Bio-Rad) are indicated on the *left*.

DNA ligase III was purified to greater than 90% homogeneity from testis nuclear extracts by monitoring formation of the 100 kDa enzyme-adenylate intermediate and joining of the oligo(dT)/poly(dA) substrate. After the final FPLC Mono Q column, a single major band with an apparent molecular mass of 100 kDa co-eluted with DNA joining activity. Analysis of the protein content of the peak fraction by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis detected a minor polypeptide with an apparent molecular mass of 87 kDa in addition to the major band at 100 kDa (Fig. 1, *lane 1*). In assays measuring enzyme-adenylate formation, labeled products of 100 and 87 kDa were generated in the same relative amounts as the polypeptides stained with Coomassie Blue (Fig. 1, *lane 2*). This 87-kDa polypeptide is probably the active proteolytic fragment of DNA ligase III described previously (2). Approximately 35 μg of the 100 kDa form of DNA ligase III were obtained from 750 g of bovine testes.

In DNA joining assays, the most highly purified fractions had a specific activity of 2 units/mg with the oligo(dT)/poly(dA) substrate and 0.2 unit/mg with the oligo(dT)/poly(rA) substrate. The value measured with the DNA/DNA substrate is similar to that obtained for homogeneous bovine DNA ligase I (2.5 units/mg) (14), recombinant human DNA ligase I (2.5 units/mg), and bovine DNA ligase II (2 units/mg) (18).

Bovine DNA Ligase III Is Recognized by the Antiserum Raised against the COOH-terminal Peptide Sequence Conserved in Eukaryotic DNA Ligases—To confirm that the putative DNA ligase III polypeptides were not derived from 125 kDa DNA ligase I by proteolysis, we performed immunoblotting experiments with the antiserum raised against homogeneous bovine DNA ligase I. As reported previously (2), this antiserum does not cross-react with partially purified DNA ligase III (Fig. 2*A*). However, both the 100- and 87-kDa DNA ligase III

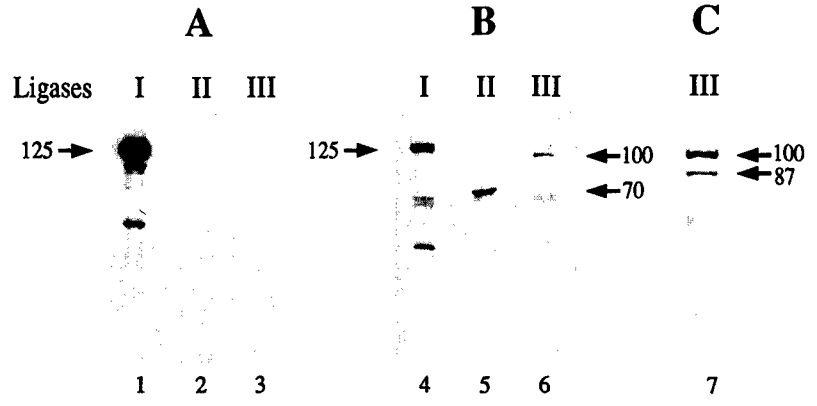

FIG. 2. **Bovine DNA ligase III cross-reacts with the antiserum raised against the conserved epitope present in all eukaryotic DNA ligases but not with an antiserum raised against bovine DNA ligase I.** Polypeptides were separated by electrophoresis through an 8% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. *A*, *lane 1*, partially purified DNA ligase I from testis whole cell extracts, 100 ng of 125-kDa polypeptide; *lane 2*, homogeneous DNA ligase II from bovine liver, 100 ng (18); *lane 3*, partially purified DNA ligase III from testis whole cell extracts, 50 ng of 100-kDa polypeptide. The membrane was incubated with antiserum raised against homogeneous DNA ligase I (14). *B*, proteins in *lanes 4–6* are identical to those in *lanes 1–3* except that the membrane was incubated with antiserum raised against a peptide common to all eukaryotic DNA ligases (14). *C*, *lane 7*, the peak fraction of DNA ligase III from testis nuclei after FPLC Mono S chromatography (400 ng). The membrane was incubated with the same antiserum as in *B*. Immune complexes were detected by enhanced chemiluminescence. The positions of the three DNA ligases are indicated. The 87-kDa band is probably an active proteolytic fragment of DNA ligase III (2).

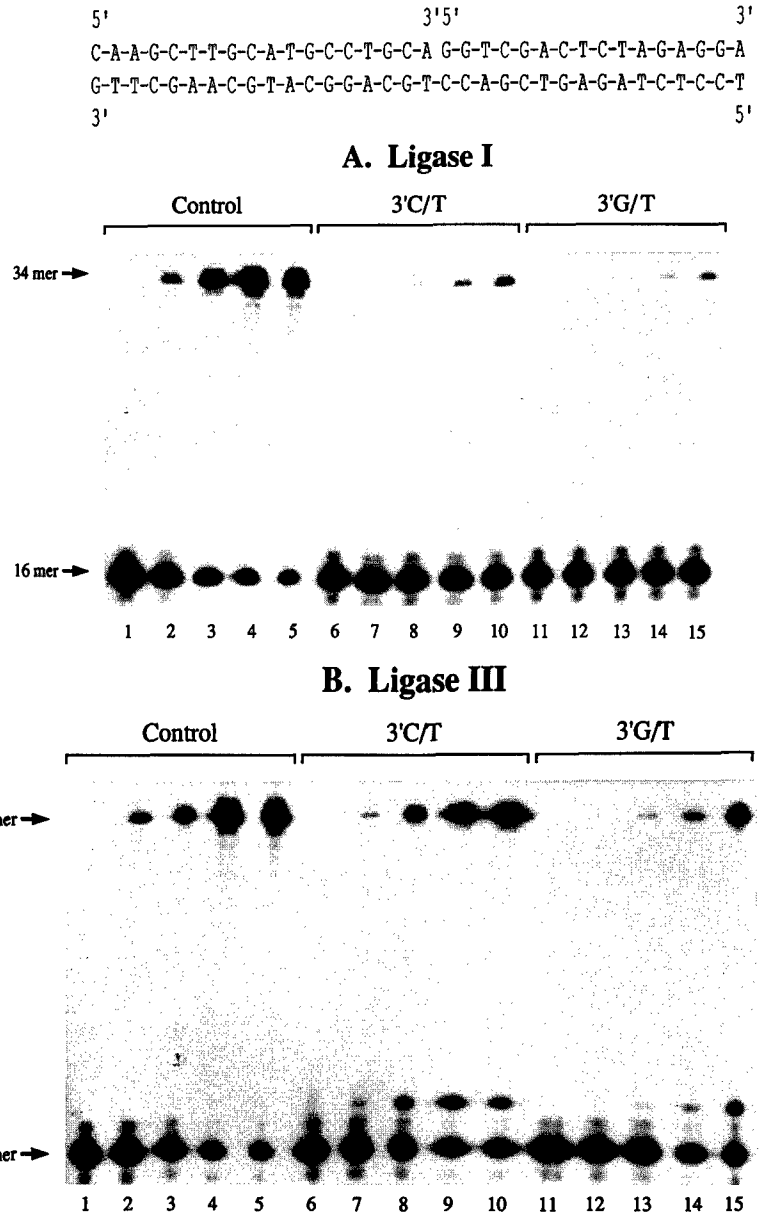

FIG. 3. **Reactivity of DNA ligases I and III with DNA substrates containing a single nick with 3′ mismatches opposite pyrimidines.** The substrates were prepared and assays performed as described under "Materials and Methods." The DNA sequence and structure of the substrate containing a single internal nick with correctly base paired termini is shown on the *top* of *A*. Similar versions of this substrate with the indicated mismatch at the 3′ terminus of the nick were constructed. In all cases, the *top right* oligonucleotide (16-mer) was labeled on the 5′ end. 3 ng of substrates was used in each reaction. *A*, joining activity of DNA ligase I with the indicated substrate. *Lane 1*, no addition; *lane 2*, 28 fmol; *lane 3*, 90 fmol; *lane 4*, 267 fmol; *lane 5*, 800 fmol of DNA ligase I added. Enzyme concentrations in *lanes 6–10* and *11–15* are the same as in *lanes 1–5*. *B*, joining activity of DNA ligase III with indicated substrate. *Lane 1*, no addition; *lane 2*, 7 fmol; *lane 3*, 21 fmol; *lane 4*, 60 fmol; *lane 5*, 180 fmol of DNA ligase III. Enzyme concentrations in *lanes 6–10* and *11–15* are the same as in *lanes 1–5*. After electrophoresis through a 10% denaturing polyacrylamide gel, labeled oligonucleotides were detected by autoradiography and quantitated by phosphorimage analysis. Although the two DNA ligases have similar specific activities in assays with the oligo(dT)/poly(dA) substrate, DNA ligase III is about 4-fold more active with the control oligonucleotide substrate.

polypeptides are recognized by the antiserum raised against a conserved COOH-terminal peptide sequence (Fig. 2, *B* and *C*) that was originally identified in a comparison of *Saccharomyces cerevisiae* Cdc9 DNA ligase, *Schizosaccharomyces pombe* Cdc17 DNA ligase and vaccinia DNA ligase (33). Subsequently, homologous peptide sequences have been found in mammalian DNA ligase I (14, 34) and DNA ligase II (18). The conservation of this peptide sequence in all eukaryotic DNA ligases presumably indicates that it plays an important but as yet undefined role in the catalytic function of these enzymes.

Reactivity of DNA Ligases I, II, and III with Polynucleotide Substrates Containing a Single Defined Nick—The three mammalian DNA ligases can be distinguished by their reactivity with different homopolymer substrates (2), but these differences in substrate specificity may not be physiologically significant. Consequently, we have examined the reactivity of the three mammalian DNA ligases with DNA molecules containing a single, defined nick that more closely resembles the *in vivo* substrate. Consistent with previous studies on *S. cerevisiae* Cdc9 DNA ligase (28), the efficiency of DNA joining by the mammalian DNA ligases was not significantly affected by 5′-mismatched termini (data not shown). Using DNA substrates with 3′-mismatched termini opposite a pyrimidine, DNA ligase III was not significantly inhibited by a 3′C/T mismatch, but a 3′G/T mismatch reduced the amount of ligated product by about 5-fold (Fig. 3*B*). DNA ligase I, however, was more severely inhibited by the same mismatches, producing 5–10-fold less ligated product than DNA ligase III (Fig. 3*A*).

Since the 3′G/T mismatch was more inhibitory than the 3′C/T mismatch, the inhibition may be due to steric effects rather than the absence of correct base pairing. Therefore, we have examined the effects of 3′-mismatched termini opposite purines. DNA joining by DNA ligase I was inhibited more than 50-fold (Fig. 4*A*). DNA ligase III was also markedly inhibited by a 3′A/G-mismatched terminus (Fig. 4*B*, *lanes 7–10*), but in contrast with DNA ligase I, the 3′T/G mismatch only reduced DNA joining by 2-fold (*lanes 12–15*). The results of assays with DNA ligase II were similar to those shown for DNA ligase III (data not shown). Thus, the inhibition of DNA joining appears to be mediated by steric hindrance, in particular by the 3′-terminal residue. However, DNA ligases II and III are much more tolerant of inappropriate 3′ termini than DNA ligase I.

Mammalian DNA Ligase III Is Closely Related to DNA Ligase II and Vaccinia DNA Ligase—A recent peptide mapping

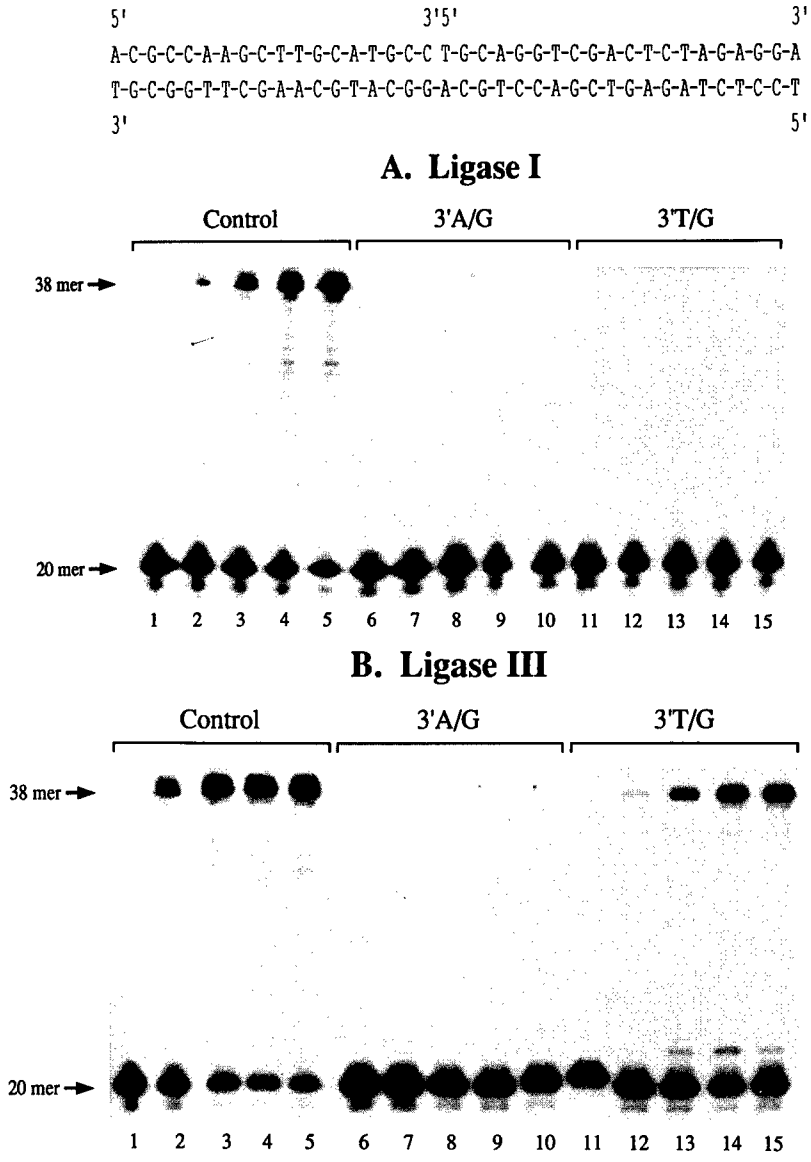

FIG. 4. **Reactivity of DNA ligases I and III with DNA substrates containing 3′ mismatches opposite purines.** The substrates were prepared and assays performed as described under "Materials and Methods." The DNA sequence and structure of the substrate containing a single internal nick with correctly base-paired termini is shown on the *top* of *A*. Similar versions of this substrate with the indicated mismatch at the 3′ terminus of the nick were constructed. In all cases, the *top right* oligonucleotide (20-mer) was labeled on the 5′ end. 3 ng of substrates was used in each reaction. *A*, joining activity of DNA ligase I with the indicated substrate. *Lane 1*, no addition; *lane 2*, 28 fmol; *lane 3*, 90 fmol; *lane 4*, 267 fmol; *lane 5*, 800 fmol of DNA ligase I added. Enzyme concentrations in *lanes 6–10* and *11–15* are the same as in *lanes 1–5*. *B*, joining activity of DNA ligase III with indicated substrate. *Lane 1*, no addition; *lane 2*, 7 fmol; *lane 3*, 21 fmol; *lane 4*, 60 fmol; *lane 5*, 180 fmol of DNA ligase III. Enzyme concentrations in *lanes 6–10* and *11–15* are the same as in *lanes 1–5*. After separation by denaturing gel electrophoresis, the production of a labeled 38-mer by ligation of a 5′ ^{32}P-labeled 20-mer to an 18-mer was detected by autoradiography and quantitated by phosphorimage analysis.

study of labeled DNA ligase-adenylate intermediates concluded that the active site regions of DNA ligases II and III are highly related (22). In an attempt to determine whether these enzymes are derived from the same gene or encoded by separate, homologous genes, we have obtained amino acid sequences from two different preparations of bovine DNA ligase III. After proteolytic digestion and separation of the resultant peptides by reverse phase HPLC, the amino acid sequences of 18 different peptides have been determined. Several peptides isolated from the two different preparations of bovine testis DNA ligase III were identical even though each preparation was purified and cleaved differently (Lys-C digestion of the near-homogenous DNA ligase III from testis nuclei and tryptic digestion of the gel-purified 100-kDa DNA ligase III from testis whole cell extract). A comparison of the 18 peptides with the predicted amino acid sequences of eukaryotic DNA ligases revealed that DNA ligase III exhibits striking homology with vaccinia DNA ligase. Out of the 18 sequences, 13 could be aligned with homologous sequences in vaccinia DNA ligase (Fig. 5). The degree of identity ranged from 30 to 86% with an overall average of 60% for the 177 residues aligned. Several of the DNA ligase III peptides could also be aligned with the catalytic domain of human DNA ligase I, exhibiting about 30% overall identity (data not shown).

As shown previously (18), peptides derived from homogeneous bovine DNA ligase II also exhibited a similar high degree of identity with vaccinia DNA ligase (Fig. 5). A comparison of DNA ligase II and DNA ligase III peptides that are homologous with vaccinia DNA ligase identified 10 peptides (136 amino acids) with identical sequences (Fig. 5). These peptide sequences encompass almost the entire predicted open reading frame of the vaccinia DNA ligase gene which encodes a 63-kDa polypeptide (33). Analysis of DNA ligase II and DNA ligase III peptides that were not homologous with vaccinia DNA ligase identified another peptide sequence, Glu-Leu-Tyr-Gln-Leu-Ser-Lys, that was common to both polypeptides, indicating that the homology between DNA ligases II and III extends beyond the bounds of vaccinia DNA ligase.

In the absence of unambiguous changes in amino acid sequence between DNA ligases II and III, it appears that these enzymes are derived from the same gene. We have not detected conversion of 100-kDa DNA ligase III into a 70-kDa active fragment during purification, arguing against nonspecific proteolysis by endogenous proteases. Furthermore, there is no evidence for a liver-specific processing mechanism, since incubation of near homogeneous DNA ligase III with liver nuclear extracts also failed to generate an active 70-kDa fragment (data not shown).

```
Vacc    MTSLREFRKLCCDIYHASGYKEKSKLIRDFITDRDDKYLIIKLLLPGLDDRIYNMNDKQI   60
                                  + ++                          ++ +++
Lig II                          TQIIQDFLQK
Lig III                         TQIIQDFLQK                    SVYNLNDK

Vacc    IKLYSIIFKQSQEDMLQDLGYGYIGDTIRTFFKENTEIRPRDKSILTLEDVDSFLTTLSS  120
                                                      + ++   ++ ++
Lig II
Lig III                                               SLLTIQEVDEFLL

Vacc    VTKESHQIKLLTDIASVCTCNDLKCVVMLIDKDLKIKAGPRYVLNAISPNAYDVFRKSNN  180
                                                     ++ +  ++++  +
Lig II
Lig III                                              VLDALDPNAYEAFK

Vacc    LKEIIENASKQNLDSISISVMTPINPMLAESCDSVNKAFKKFPSGMFAEVKYDGERVQVH  240
                                                       + +++ + ++++++++++
Lig II                                               CPNGMFSEIKYDGERVQVH
Lig III

Vacc    KNNNEFAFFSRNMKPVLSHKVDYLKEYIPKAFKKATSIVLDSEIVLVDEHNVPLPFGSLG  300
            +  +++  ++++        +  ++ ++    +  ++++  +
Lig II   KGDHFSYFSRSLK          KDFIPIAFPGGHSMILDSEVLL
Lig III  KGDHFSYFSRSLKPVLP       DFIPIAFPGGHSMILDSEVLLI-N

Vacc    IHKKKEYKNSNMCLFVFDCLYFDGFDMTDIPLYERRSFLKDVMVEIPNRIVFSELTNISN  360
                 + +++++++ ++       + ++ +    + + ++++++ + +++
Lig II      AAFQDANVCLFVFDCIYFNDVSLMDRPL-E   LLHDNMVEIPNAIMFSEMK
Lig III                                     LLHDNMVEIPNAIMFSEM

Vacc    ESQLTDVLDDALTRKLEGLVLKDINGVYEPGKRRWLKIKRDYLNEGSMADSADLVVLGAY  420
                               + ++++++ +++ + ++++++ +++ ++++++++
Lig II                         GTYEPGKRHWLK   DYLNEGAMADTADLVVLGAF
Lig III                              RHWLK-K DYLNEGAMADTADLVVLGAF

Vacc    YGKGAKGGIMAVFLMGCYDDESGKWKTVTKCSG HDDNTLRVLQDQLTMVKINKDPKKIP  479
        ++ + +++ +  +++++++  + ++ +++++ + +++ ++  ++  + +++        ++
Lig II  YGQGSKGGMMSIFLMGCYDPSSQKWCTVTKCAGGHDDATLARLQ-ELDMVK        IP
Lig III Y-Q                     WCTVTKCAG-HDDA-LARLQREL-MVK

Vacc    EWLVVNKIYIPDFVVEDPKQSQIWEISGAEFTSSKSHTANGISIRFPRFTRIREDKTWKE  539
         ++    ++ +++ + +++    +++ ++++  +  +++ ++++++++ + +
Lig II  NWLK   IYYPDFIVPDPK AAVW-ITGA-FS SEAHTADGISIRFPR-T-I
Lig III        IYYP-FIVPDP  AAVWEITGAEF-K

Vacc    STHLNDLVNLTKS                                                  552
Lig II
Lig III
```

FIG. 5. **Alignment of the peptide sequences from DNA ligases II and III with vaccinia DNA ligase.** The peptide sequences from bovine DNA ligase II have been reported previously (18) except for the peptide TQIIQDFLQK. These sequences and peptide sequences from bovine DNA ligase III have been aligned with the predicted amino acid sequence of vaccinia DNA ligase (1–552) (33). A single gap has been introduced for maximum alignment. A *hyphen* indicates a position within a peptide where it was not possible to assign an amino acid. The 6-residue DNA ligase active site motif (29) is indicated in *boldface*. The sequence of the DNA ligase II peptide, CAGGHDDATLARLQELDMVK (18), has been modified after reexamination of the amino acid sequencing data and comparison with the homologous DNA ligase III peptide. The COOH-terminal residue of each peptide is *underlined*. In the absence of unambiguous changes in sequence between homologous peptides from DNA ligase II and III, only amino acids conserved between the peptides and vaccinia DNA ligase are marked with a *cross*.

Irrespective of the exact relationship between DNA ligases II and III, it appears that there are two distinct families of eukaryotic DNA ligases, which probably evolved from a common ancestral gene (Fig. 6). One family consists of mammalian DNA ligase I, *Drosophila* DNA ligase I (35), *S. cerevisiae* Cdc9 DNA ligase (36), and *S. pombe* Cdc17 DNA ligase (37). The primary function of these enzymes is to join Okazaki fragments during DNA replication. The second family consists of mammalian DNA ligases II (18) and III, *Drosophila* DNA ligase II (38), and the DNA ligases encoded by vaccinia and other pox viruses (33, 39, 40). These enzymes are probably involved in DNA repair and/or genetic recombination pathways.

DISCUSSION

DNA ligase III has been purified to >90% homogeneity from bovine testis nuclei. We have concluded that the major 100-kDa polypeptide detected by Coomassie Blue staining in the most highly purified fractions is DNA ligase III for the following reasons: (i) the 100-kDa polypeptide cross-reacts with an antiserum raised against a peptide sequence found in all eukaryotic DNA ligases; (ii) the amino acid sequences of peptides derived from the 100-kDa polypeptide exhibit striking homology with the coding sequences of other eukaryotic DNA ligases; (iii) in the presence of labeled ATP, a 100-kDa labeled enzyme-adenylate complex is formed; (iv) in DNA joining assays, the

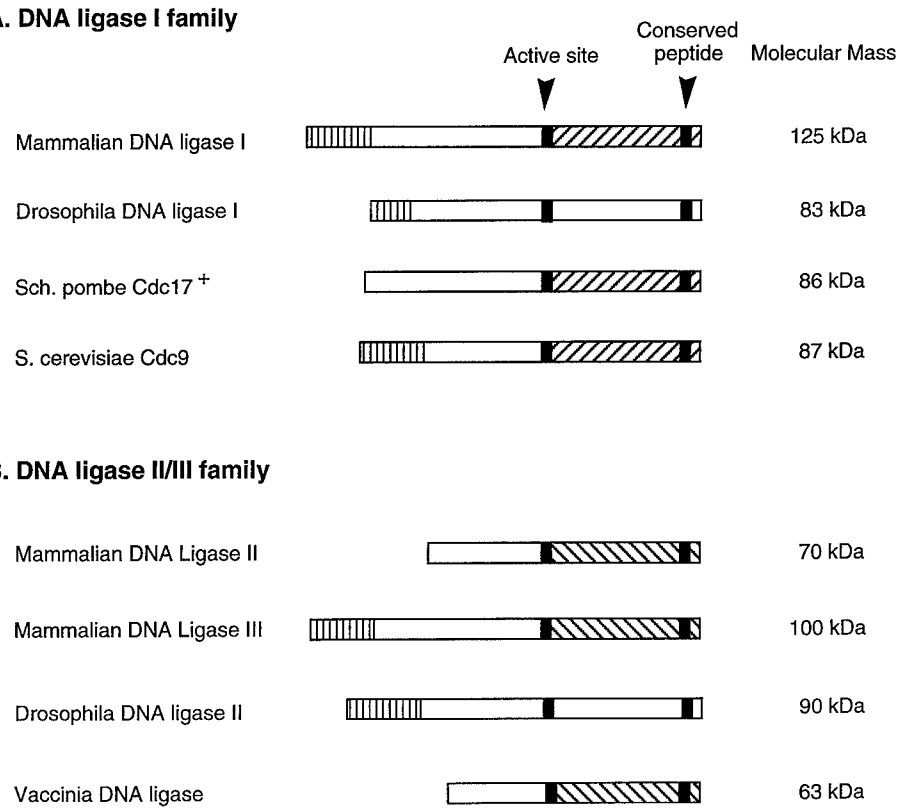

FIG. 6. **Two families of ATP-dependent DNA ligases in eukaryotes and eukaryotic viruses defined by similarities in amino acid sequence and/or biochemical properties.** The molecular mass of the DNA ligases, measured by denaturing gel electrophoresis are shown on the *right*. The *areas filled in* with *vertical lines* indicate regions of the enzymes that have been demonstrated to be dispensable for catalytic activity. The known or predicted positions of the active site motif containing the reactive lysine residue (18, 29) and of the conserved peptide sequence (14, 33) are indicated by *arrows*. The *areas filled in* with *diagonal lines* represent regions that share 50% or higher amino acid identity. *A*, mammalian DNA ligase I (14, 29, 34), *Drosophila* DNA ligase I (35), *S. pombe* Cdc17 DNA ligase (37), and *S. cerevisiae* Cdc9 DNA ligase (28, 36). *B*, mammalian DNA ligase II (18, 22), mammalian DNA ligase III (2, 22), *Drosophila* DNA ligase II (38), and vaccinia DNA ligase (33).

specific activity of DNA ligase III is similar to that of homogeneous DNA ligases I and II.

High levels of both DNA ligase I and DNA ligase III activity were present in whole cell extracts from testes. During spermatogenesis, diploid germ cells replicate their genome to generate a cell with a DNA content of 4 N prior to the two meiotic divisions. Mouse germ cells undergoing premeiotic DNA synthesis contain high levels of DNA ligase I activity (32), indicating that DNA replication in germ cells is carried out by the same enzymes that function in somatic cells (5, 17). We suggest that the elevated levels of DNA ligase III reflect the involvement of this enzyme in meiosis. A potential role for DNA ligase III during meiosis would be to complete the large number of homologous recombination events that precede the first meiotic cell division.

In the life cycle of the yeast *S. cerevisiae*, sporulation is functionally equivalent to gametogenesis in mammals. After transfer to sporulation media, expression of the *CDC9* DNA ligase gene, whose product is functionally homologous to mammalian DNA ligase I (28, 34), is induced prior to the premeiotic S phase (41). After DNA replication, the cells proceed through the first meiotic division with mature recombinants arising at the end or just after pachytene (42). Genes in the *RAD52* epistasis group were initially isolated, because mutations confer sensitivity to ionizing radiation (43). Further analysis of these mutants has demonstrated that they are defective in meiosis (44) in addition to DNA strand break repair. The high levels of DNA ligase III in the testes, the association of DNA ligase III with the product of the human strand break repair gene *XRCC1* (24), which is also expressed at high levels in testes (45), and the decreased levels of DNA ligase III in a xrcc1 mutant cell line EM9, which is defective in DNA strand break repair (24, 46), are consistent with DNA ligase III also being involved in both meiotic recombination and DNA strand break repair.

The three mammalian DNA ligases were distinguished by their reactivity with different homopolymer substrates (2). We have investigated the ability of these enzymes to seal nicks with mismatched termini. The substrate specificities of DNA ligases II and III were similar, whereas the substrate specificity of recombinant human DNA ligase I was identical with that of Cdc9 DNA ligase (28). Thus, the family of functionally homologous replicative DNA ligases appear to be much more sensitive to inhibition by 3′ mismatches than the family of DNA ligases that includes DNA ligases II and III and the poxvirus DNA ligases. This may indicate that a stringent enzyme is required to join Okazaki fragments during DNA replication. In contrast, the ability to join nicks with 3′-mismatched termini may be tolerated or preferred in certain DNA repair and recombination pathways.

The differences in amino acid sequence of adenylylated peptides from DNA ligase I and DNA ligase II confirmed that these enzymes are encoded by different genes (18). The isolation of identical peptides from apparently homogenous preparations of DNA ligases II and III indicates that these enzymes are encoded either by the same gene or by two highly homologous genes. Based on the alignment with vaccinia DNA ligase, the DNA ligase II peptides are distributed over a region of 55 kDa. If we assume that the 70-kDa DNA ligase II consists of 640 amino acids then the 136 amino acids that are identical with DNA ligase III represent 21% of DNA ligase II. Although we cannot exclude the possibility that there are differences in other regions of these polypeptides, the absence of significant differences in amino acid sequence between homologous peptides suggests that these enzymes are probably derived from the same gene. This conclusion is consistent with a recent study which demonstrated that the catalytic domains of DNA ligases II and III are highly related (22).

We do not believe that DNA ligases II is an active proteolytic fragment of DNA ligase III that is generated by proteolysis during purification for the following reasons: (i) the 70-kDa DNA ligase II polypeptide was blocked to Edman degradation, indicating that it possessed the modified amino-terminal residue of the primary translation product (18); (ii) conversion of

DNA ligase III to an active fragment similar in size to DNA ligase II has not been observed following incubation of DNA ligase III either with liver nuclear extracts or proteases (2, 22); (iii) DNA ligase II and DNA ligase III are present at different levels in different mammalian tissues (2, 18); (iv) the mutant Chinese hamster ovary cell line, EM9 has reduced levels of DNA ligase III activity but normal levels of DNA ligase II activity (24, 46, 47).

Based on similarities in amino acid sequence and/or polynucleotide substrate specificity, the DNA ligases of eukaryotes and eukaryotic viruses can be grouped into two families. This grouping also appears to reflect cellular function. Within the first family, mammalian DNA ligase I, *S. cerevisiae* Cdc9 DNA ligase and *S. pombe* Cdc17 DNA ligase have all been shown to be required for DNA replication. The cellular functions of the second family, which consists of mammalian DNA ligases II and III, *Drosophila* DNA ligase II, and the poxvirus DNA ligases, have been less clearly defined. Vaccinia virus DNA ligase is not required for viral replication, does not affect viral recombination, but influences the sensitivity of the virus to DNA damage (48, 49). This suggests that vaccinia DNA ligase functions in DNA repair. A similar role has been proposed for DNA ligase II (20, 21). The high levels of DNA ligase III in the testes, its association with a thymus recombination complex (23), and its interaction with a DNA strand break repair protein (24, 46) implicate this enzyme in both DNA repair and genetic recombination. We suggest that the DNA ligases in this second family have evolved to fulfill specific functions in pathways of DNA repair and genetic recombination.

In summary, we have purified DNA ligase III to near physical homogeneity from bovine testes. The high level of DNA ligase III in this tissue suggests a role for this enzyme in germ cell development, specifically during meiosis. Amino acid sequencing studies demonstrate that DNA ligase III is highly homologous with vaccinia DNA ligase and appears to be identical with DNA ligase II. The availability of amino acid sequence information from DNA ligase II (18) and DNA ligase III should facilitate the cloning of the gene(s) coding for the two enzymes. This in turn will permit further investigation of their relationship and their respective roles in mammalian DNA metabolism.

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Mammalian DNA Ligase III: Molecular Cloning, Chromosomal Localization, and Expression in Spermatocytes Undergoing Meiotic Recombination

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Three biochemically distinct DNA ligase activities have been identified in mammalian cell extracts. We have recently purified DNA ligase II and DNA ligase III to near homogeneity from bovine liver and testis tissue, respectively. Amino acid sequencing studies indicated that these enzymes are encoded by the same gene. In the present study, human and murine cDNA clones encoding DNA ligase III were isolated with probes based on the peptide sequences. The human DNA ligase III cDNA encodes a polypeptide of 862 amino acids, whose sequence is more closely related to those of the DNA ligases encoded by poxviruses than to replicative DNA ligases, such as human DNA ligase I. In vitro transcription and translation of the cDNA produced a catalytically active DNA ligase similar in size and substrate specificity to the purified bovine enzyme. The DNA ligase III gene was localized to human chromosome 17, which eliminated this gene as a candidate for the cancer-prone disease Bloom syndrome that is associated with DNA joining abnormalities. DNA ligase III is ubiquitously expressed at low levels, except in the testes, in which the steady-state levels of DNA ligase III mRNA are at least 10-fold higher than those detected in other tissues and cells. Since DNA ligase I mRNA is also present at high levels in the testes, we examined the expression of the DNA ligase genes during spermatogenesis. DNA ligase I mRNA expression correlated with the contribution of proliferating spermatogonia cells to the testes, in agreement with the previously defined role of this enzyme in DNA replication. In contrast, elevated levels of DNA ligase III mRNA were observed in primary spermatocytes undergoing recombination prior to the first meiotic division. Therefore, we suggest that DNA ligase III seals DNA strand breaks that arise during the process of meiotic recombination in germ cells and as a consequence of DNA damage in somatic cells.

The pleiotropic effects of mutations in the DNA ligase gene of prokaryotes include conditional lethality, sensitivity to DNA damage, and hyperrecombination (29). At the nonpermissive temperature, DNA ligase mutants are unable to join Okazaki fragments and consequently cannot complete DNA replication. The sensitivity to DNA damage arises from the impaired ability to seal DNA strand breaks generated either directly by the DNA damaging agent or by DNA repair enzymes excising lesions. In contrast to prokaryotes, multicellular eukaryotes contain more than one species of DNA ligase (50, 54, 55). One of these enzymes, DNA ligase I, is required for DNA replication (4, 49, 58) and also appears to be involved in DNA repair (38). The biochemically distinct DNA ligases II and III have been less extensively studied. The levels of DNA ligase II activity, the major DNA joining enzyme in the liver (49, 60), are increased following treatment with DNA-damaging agents, suggesting a role for this enzyme in DNA repair (11, 14). DNA ligase III has been identified as a component of a calf thymus recombination complex (24) and has been found associated with a human DNA strand break repair protein, Xrcc1 (8), suggesting roles for this enzyme in genetic recombination and DNA repair.

Recently, we purified 70-kDa DNA ligase II and 100-kDa DNA ligase III to near homogeneity from bovine liver tissue and bovine testis tissue, respectively. Amino acid sequencing studies revealed that these polypeptides share extensive regions of identity, indicating that they are probably encoded by the same gene (23, 60). A comparison of the peptide sequences from DNA ligases II and III with the predicted amino acid sequences of other DNA ligases revealed that these enzymes are more highly related to the DNA ligases encoded by cytoplasmic poxviruses, in particular vaccinia virus DNA ligase, than to mammalian DNA ligase I and other replicative DNA ligases (23, 60). Interestingly, deletion of the poxvirus DNA ligase does not affect viral DNA replication or recombination but renders the mutant virus more sensitive to DNA damage (12, 27).

Defects in DNA joining have been described for cell lines derived from patients with the hereditary cancer-prone disease Bloom syndrome (BLM) (30, 41) and also for the cell line 46BR and its derivatives that were established from a patient with severe combined immunodeficiency (20, 31, 38). The symptoms of this patient appear to be caused by mutations in

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the DNA ligase I gene (5). In contrast, the absence of DNA ligase I mutations in representative BLM cell lines and the localization of the DNA ligase I and BLM genes to different chromosomes have eliminated this gene as a candidate for BLM (4, 5, 32, 36). The previously observed alterations in high-molecular-weight DNA ligase activity, partially purified from BLM cell extracts (10, 61, 62), may be caused either by mutations in the DNA ligase III gene or by mutations in a gene whose product interacts with and regulates DNA ligase I or DNA ligase III activity.

Since meiosis is a unique developmental process in the life cycle of sexually reproducing eukaryotes, a different repertoire of DNA metabolic enzymes, such as a DNA ligase, may be required to perform the meiosis-specific DNA transactions. A characteristic feature of meiosis is the high frequency of homologous recombination that leads to the production of genetically reassorted haploid gametes. These recombination events, which permit the correct segregation of homologous chromosomes in the first meiotic division, appear to occur within the synaptonemal complex (18, 35, 52) and presumably are completed by DNA synthesis and DNA ligation prior to the first meiotic division (51).

In this report, we describe the molecular cloning of human and murine DNA ligase III cDNAs. The human gene, which is present on human chromosome 17, appears to be ubiquitously expressed. Consistent with previous biochemical studies (23), the highest steady-state levels of DNA ligase III mRNA are found in the testes. During spermatogenesis, DNA ligase I is highly expressed in spermatogonia whereas the highest levels of DNA ligase III expression occur in primary spermatocytes. These observations are consistent with DNA ligase I functioning in premeiotic DNA replication and with DNA ligase III completing meiotic recombination events.

MATERIALS AND METHODS

Materials and general methods. Standard molecular biology techniques were performed as described elsewhere (44). Human liver 5′-rapid amplification of cDNA ends (RACE)-ready cDNA, human testis λgt10 5′-stretch plus and λDR2 5′-stretch cDNA libraries, mouse testis λgt10 5′-stretch cDNA library, λgt10 and pDR2 PCR primers, human and mouse multiple tissue Northern (RNA) blots, and adult testis poly(A)$^+$ RNA were purchased from Clontech. Restriction enzymes were purchased from New England BioLabs. [α-^{32}P]dCTP and [α-^{32}P]ATP were purchased from Amersham. The plasmid vectors pBluescript II SK$^+$ and pGEM3Z were from Stratagene and Promega, respectively. Unless otherwise indicated, the vector used for all of the cloning was pBluescript II SK$^+$. Plasmid DNA was routinely isolated by using Mini and Maxi Wizard plasmid preparation kits (Promega). Transformation was performed with library-efficiency *Escherichia coli* DH5αF′ competent cells from GIBCO BRL. DNA sequencing primers were synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer. Degenerated oligonucleotides were synthesized and purified by Operon Technologies, Inc., and Genesys Inc. Paraffin-embedded sections of mouse testes were obtained from Novagen. All chemicals were of molecular biology grade.

Peptide sequences from bovine DNA ligases II and III. The amino acid sequences of 16 DNA ligase II peptides and 13 DNA ligase III peptides have been reported previously (23, 60). An additional four DNA ligase II and five DNA ligase III peptides have been obtained and are listed in the legend to Fig. 1.

Isolation of DNA ligase III-specific probe by degenerate PCR. Degenerate oligonucleotides were designed on the basis of bovine DNA ligase III peptides sequences (23) which were aligned on the vaccinia virus DNA ligase sequence. The following amino acid regions were selected: primer 1, T I Q E V D E F [sense strand; ACIAT(T/A/C)CA(A/G)GA(A/G)GTIGA(T/C)GA(A/G)TT]; primer 2, KGDHFSYF [antisense strand; AA(A/G)TAI(C/G)(T/A)(A/G)AA(A/G)TG(A/G)TCICC(T/C)TT]; primer 3, NEGAMAD [sense strand; AA(T/C)GA(A/G)GG(G/A/T/C)GC(G/A/T/C)ATGGC(G/A/T/C)GA]; and primer 4, QKWCTVT [antisense strand; GT(G/A/T/C)AC(G/A/T/C)GT(G/A)CACCA(T/C)TT(T/C)TG]. Standard PCRs (43) were carried out. A typical reaction mixture (50 μl) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 10^7 λ phage, and 0.2 mM of each primer (primers 1 and 2). After incubations at 94°C for 5 min and at 52°C for 5 min, 2.5 U of *Taq* polymerase (Promega) was added to initiate the reaction. After incubation at 72°C for 1.5 min, PCR amplification was carried out by 30 cycles of 94°C for 0.5 min, 52°C for 2 min, and 72°C for 1.5 min after which there was a 5-min incubation at 70°C. The anticipated 460-bp fragment (based on the homology between the peptides and vaccinia virus DNA sequence) was subcloned and sequenced. The deduced amino acid sequence of the 460-bp fragment was 50% identical with vaccinia virus DNA ligase and 100% identical with the DNA ligase III peptide, VLDALDPNAYEAFK (23), and the DNA ligase II active-site peptide, CPNGMFSEIKYDGERVQVH (60).

An oligo(dT)-primed first-strand cDNA was prepared from HeLa poly(A)$^+$ RNA by using the Reverse Transcription system (Promega). The HeLa cDNA was amplified by PCR with primers 3 and 4 under the conditions described above. The anticipated 150-bp fragment was subcloned into *Sma*I-digested pGEM3Z. The deduced amino acid sequence of the 150-bp fragment was identical to those of the bovine peptides except for one conservative change (60).

Cloning of human DNA ligase III cDNA. The 460-bp fragment was used as a probe to screen a human testis cDNA library. A total of 5×10^5 plaques (2.5×10^4 per plate) was transferred to MagnaGraph nylon transfer membranes (Micron Separations Inc.) and hybridized with the labeled 460-bp probe (10^9 to 10^{10} cpm/μg of DNA) according to the manufacturer's protocol. The filters were then washed sequentially in 3× SSC (1× SSC is 8.77 g of NaCl plus 4.41 g of sodium citrate per liter [pH 7.0])–0.5% sodium dodecyl sulfate (SDS) at room temperature (RT) for 20 min twice, in 1× SSC–0.5% SDS at 40°C for 20 min twice, and in 1× SSC–0.5% SDS at 50°C for 30 min. Additional rounds of screening were carried out to isolate a homogeneous phage population. The size of the cDNA insert was initially determined by PCR with primers flanking the *Eco*RI cloning site of λgt10. After preparation of DNA from homogeneous phage populations, the cDNA inserts were excised by digestion with *Eco*RI and analyzed by agarose gel electrophoresis. These cDNAs were also screened for hybridization with the 150-bp PCR probe by Southern blotting. The cDNA inserts that hybridized with both PCR probes were subcloned into pBluescript II SK$^+$. The largest clone (2.9 kb) contained an internal *Eco*RI site. Both *Eco*RI fragments were cloned into M13mp19 vector in two orientations for sequencing analyses.

Analysis of the 5′ and 3′ ends of human DNA ligase III cDNA by RACE and genomic PCRs. During sequencing of several cDNA clones, we noted a difference in the number of A residues starting at nucleotide 552 (Fig. 1). Independent cDNA clones contained either 8, 9, or 17 A residues at this position but no differences in the flanking sequences. The open reading frame (ORF) in sequences containing 8 or 17 A residues encodes an amino acid sequence that is identical to the bovine peptide IEDLTELE immediately after this A track. 5′-RACE PCR (16) was performed by using a human liver 5′-RACE-ready cDNA. Two nested antisense primers complementary to the regions 702 to 677 and 669 to 647 were made, and the PCR was carried out as suggested by the manufacturer. A single major 600-bp fragment was amplified. This fragment was subcloned into the vector, and the sequences of six independent clones were determined. The sequences of all six clones were essentially identical to the sequence shown in Fig. 1 (nucleotides 55 to 646), except for heterogeneity at the A tract. The number of A residues varied from 6 to 19.

We considered the possibility that the A tract heterogeneity was an artifact introduced by reverse transcriptase. Therefore, we amplified the corresponding sequence from human genomic DNA by PCR with an oligonucleotide corresponding to nucleotides 484 to 510 and an oligonucleotide complementary to nucleotides 582 to 562 of the cDNA sequence (Fig. 1). The expected PCR product of 99 bp was isolated after gel electrophoresis and subcloned into pBluescript SK$^+$. A run of eight A residues was found in the DNA sequences of three independent clones, indicating that this is the correct sequence.

The ORF encoded by the largest cDNA isolated from the human testis library did not contain a stop codon. Therefore, we amplified the 3′ end of this cDNA from an oligo(dT)-primed human testis λDR2 cDNA library by PCR with *Pfu* DNA polymerase (Stratagene). After an initial PCR with pDR2 3′-AMP sequencing primer and a primer corresponding to nucleotides 2417 to 2442 (Fig. 1), an aliquot of the reaction mixture was reamplified with pDR2 3′-AMP sequencing primer and a primer corresponding to nucleotides 2444 to 2469 (Fig. 1). After separation by agarose gel electrophoresis, the major amplified product of 600 bp was cloned and four individual clones were sequenced. Within this sequence, which extended the original cDNA by 200 nucleotides, there was an in-frame stop codon terminating the DNA ligase III ORF 89 nucleotides from the poly(A) tail (Fig. 1). A full-length DNA ligase III cDNA (*LIG3*) was constructed from the large *Eco*RI fragment containing the 8-residue A sequence and the 600-bp *Eco*RI-*Xba*I 3′-RACE PCR product.

Cloning of mouse DNA ligase III cDNA. A 1.96-kb fragment of human DNA ligase III cDNA (nucleotides 490 to 2452) was used as a probe to screen a mouse testis cDNA library (λgt10 mouse testis 5′-stretch cDNA library) essentially as described above. A full-length and several partial cDNA clones that exhibit 80 to 90% sequence homology with the human DNA ligase III cDNA have been identified. Murine DNA ligase III cDNA fragments corresponding to nucleotides 296 to 960, 2034 to 2452, and 1430 to 2452 of human DNA ligase III cDNA (Fig. 1) have been employed in further studies.

DNA sequencing and sequence analysis. Single- and double-strand DNA sequencing were carried out by the dideoxy chain termination method (45) with Sequenase (U.S. Biochemical) and synthetic sequencing primers. The DNA sequences of both strands were determined with primers at intervals of about 150 bases. Sequence translations and peptide alignments were performed with IntelliGenetics. Data base (NCBI-GenBank) searches were performed at the Na-

tional Center for Biotechnology with the basic local alignment search tool (BLAST) network service (1). Protein sequence homologies were aligned with DNAStar MegAlign by the CLUSTAL method (22). Multiple alignment parameters were a gap penalty of 10 and a gap length of 10. Pairwise alignment parameters were a ktuple of 1, a gap penalty of 3, a window of 5, and diagnols saved of 5. A phylogenetic tree was also constructed with the same program (42).

In vitro translation of human DNA ligase III cDNA: reactivity of in vitro-translated DNA ligase III. The full-length DNA ligase III clone (*LIG3*) and two 3′ deletions were subcloned into pBluescript SK$^+$ under the control of the T7 promoter. Coupled in vitro transcription and translation (Promega) reactions (50-μl mixtures) were carried out with 0.5 to 2 μg of plasmid DNA, 40 μCi of [^{35}S]methionine, and T7 RNA polymerase at 30°C for 90 min according to the manufacturer's protocol. For detecting enzyme activity, the in vitro translation reaction was performed in the absence of [^{35}S]methionine. After partial purification by $(NH_4)_2SO_4$ precipitation (3), in vitro-translated products were resuspended to the original volume in a buffer containing 50 mM Tris-HCl (pH 7.5), 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2 μg of aprotinin per ml, 2 μg of chymostatin per ml, 1 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 2 mM Pefabloc, 20 μg of TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) per ml, 10 μg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone) per ml, and 100 μg of trypsin inhibitor per ml. This resuspension was then spin dialyzed through a 1-ml Sephadex G-25 column equilibrated in the same buffer. Aliquots (0.5 to 1.5 μl) were incubated with 7.5 μCi of [α-^{32}P]ATP (~3,000 Ci/mmol) in a volume of 25 μl for 15 min as described previously (55).

To demonstrate that the labeled polypeptide-adenylate complexes were intermediates in the DNA ligation reaction, these complexes were further incubated with 20 nmol of sodium PP_i, 0.5 μg of oligo(pdT)-poly(dA), and 0.5 μg of oligo(pdT)-poly(rA) for 2 h at 20°C (55). The reactions were terminated by the addition of SDS loading buffer. Samples were electrophoresed through an SDS-8% polyacrylamide gel. The gel was washed in water for 30 to 60 min, dried, and exposed to X-ray films.

Southern hybridization. The somatic cell hybrid panel (BIOS Laboratory, Inc.) was used to map the DNA ligase III gene to a specific human chromosome. Nylon filters containing *Pst*I-digested genomic DNAs (8 μg) from each of the somatic cell hybrids and representative mouse, human, and Chinese hamster ovary cell lines were hybridized with a labeled fragment of DNA ligase III cDNA (nucleotides 1 to 809). The hybridization was carried out overnight at 65°C in 0.5 M sodium phosphate (pH 7.2)–7% SDS–1 mM EDTA–100 μg of sheared denatured salmon sperm DNA per ml. The filters were washed in 0.2× SSC–0.2% SDS twice at RT for 10 min each and twice at 65°C for 3 to 5 min each and were exposed to X-ray films.

PCR mapping. The National Institute of General Medical Sciences (NIGMS) Human-Rodent Somatic Cell Hybrid Mapping Panel 2 consists of mouse-human or hamster-human hybrids, each of which bears a single human chromosome, with the exception of the chromosome 1 and chromosome 20 hybrids, which have also retained chromosomes X and 4, respectively. Genomic DNA (200 ng) from the panel, from a human tumor cell line (HT1080), and from mouse cell lines with and without human chromosome 17 (32) were amplified by PCR with 1 μM each primer (forward primer, nucleotides 1214 to 1236; backward primer, nucleotides 1412 to 1392), 250 μM dNTPs, 0.01% gelatin, 1.5 mM $MgCl_2$, 1× PCR buffer (Perkin-Elmer), and 2.5 U of *Taq* polymerase in a 100-μl volume. Reactions were initially denatured for 2 min at 94°C and then subjected to 35 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Instead of the 198-bp fragment predicted from the cDNA sequence, the primers amplified an 850-bp fragment from human genomic DNA, indicating the presence of an intron.

Northern (RNA) hybridization. Poly(A)$^+$ RNA was prepared from the testes of 5-, 8-, 15-, and 25-day-old mice (C57BL/6J from Jackson Laboratories). Briefly, the testes were homogenized in 0.2 M NaCl–0.2 M Tris-HCl (pH 7.5)–1.5 mM $MgCl_2$–2% SDS–200 mg of proteinase K per ml. Subsequently, poly(A)$^+$ RNA was purified according to the method described by Badley et al. (2). Approximately 2 μg of each poly(A)$^+$ RNA sample was electrophoresed through a 1.2% agarose-formaldehyde gel, transferred to a nitrocellulose membrane, and then immobilized on the membrane by UV cross-linking.

The membrane was incubated in prehybridization buffer (50% formamide, 4× SSC, 50 mM sodium phosphate [pH 7.0], 100 μg of salmon sperm DNA per ml, 1% SDS) for 2 h at 42°C prior to the addition of the DNA probe (2×10^9 cpm/μg) that had been labeled with [α-^{32}P]dCTP by using the *redi*prime random primer labeling kit (Amersham). After further incubation at 42°C for 12 h, the membrane was washed once with 2× SSC–0.1% SDS for 10 min at room temperature and twice with the same buffer at 60°C for 15 min. The membrane was then exposed to X-ray film at −80°C. The hybridization of probes to commercial human and mouse multiple tissue Northern blots and the subsequent washing of the membranes were performed under the conditions recommended by the manufacturer. Labeled probes were stripped from the membranes by incubation in sterile water containing 0.5% SDS at 90°C. Poly(A)$^+$ RNA loading differences were normalized by probing membranes with GAPDH or β-actin cDNA.

Riboprobe synthesis. Digoxigenin-labeled riboprobe was prepared and quantitated as described in the Genius System User's Guide (Boehringer Mannheim). Mouse DNA ligase III cDNA fragments were cloned into the *Eco*RI site of pBluescript II SK$^+$. Sense and antisense transcripts were generated by using T3 and T7 RNA polymerases. To allow diffusion of the probe into the tissue, the size of the riboprobe was reduced to approximately 150 bases by alkaline hydrolysis.

In situ localization of DNA ligase III expression in sections of mouse testes. After being washed twice with xylene for 10 min, twice with absolute ethanol for 5 min, and once with phosphate-buffered saline (PBS) for 2 min at RT, the mouse testis sections were permeabilized by incubating the slides for 15 min in 0.3% Triton X-100 in PBS. The sections were washed with PBS and then acetylated by incubation for 10 min in 0.1 M triethanolamine hydrochloride (pH 8) containing 0.25% acetic anhydride. The slides were washed with RNase-free water and allowed to dry. The riboprobe was dissolved in 1 to 5 μl of RNase-free water, heated at 60°C for 5 min, and then added to 50 μl of hybridization mixture (50% formamide, 6× SSPE [0.9 M NaCl, 60 mM sodium dihydrogen phosphate, 6 mM EDTA; pH 7.4], 5× Denhardt's solution, 0.5% SDS, and 100 μg of freshly denatured salmon sperm DNA per ml) prior to application to the sections. The slides were incubated with the riboprobe-hybridization mixture overnight at 42°C in a humidified chamber. After hybridization, the slides were washed twice with prewarmed 1× SSPE–0.1% SDS at 42°C for 10 min and with buffer A (100 mM Tris-HCl [pH 7.5], 150 mM NaCl) for 5 min at RT. After being blocked with 2% sheep serum and 0.3% Triton X-100 in buffer A for 1 h at 37°C, the slides were incubated with alkaline phosphatase-tagged antidigoxigenin (Boehringer Mannheim; diluted 1:100 in buffer A containing 1% sheep serum and 0.15% Triton X-100) for 1.5 h at 37°C. The slides were washed three times for 5 min each with buffer A and with 100 mM Tris-HCl (pH 9)–150 mM NaCl–50 mM $MgCl_2$. Antigen-antibody complexes were visualized by using an acid fuchsin substrate development kit (Dako, Inc.). Finally, the slides were counterstained with hematoxylin (Fisher Scientific) and analyzed under a microscope. The different cell types within the seminiferous tubules were identified on the basis of their positions within the tubule, their morphological characteristics (in particular cell size and chromatin structure), and the distribution of cell types within a particular tubule (6).

RESULTS

Isolation of human DNA ligase III cDNA. To specifically amplify cDNA fragments encoding DNA ligase III by the PCR, we designed two sets of degenerate oligonucleotide primers based on peptide sequences obtained from bovine DNA ligase III. DNA fragments of the anticipated size, 150 and 460 bp, were specifically amplified from human cDNAs. The predicted amino acid sequences encoded by these fragments were >95% identical with peptides from bovine DNA ligase III (23) and about 50% identical with homologous regions of vaccinia virus DNA ligase (48). By using the 460-bp fragment as a probe, 22 independent clones with insert sizes ranging from 0.8 to 2.9 kb were isolated from a human testis cDNA library.

The longest cDNA molecule (2,843 bp) did not contain a polyadenylation signal (39) or poly(A) tail, indicating that this is not a full-length cDNA. Furthermore, we have cloned a murine DNA ligase III cDNA with a poly(A) tail that is 200 bp longer than the human DNA ligase III cDNA at the 3′ end (data not shown). The 3′ end of human DNA ligase III cDNA was amplified from a human testis cDNA library by RACE PCR. This amplified fragment extended the original sequence by 200 nucleotides to yield a full-length cDNA (*LIG3*) of 3,029 nucleotides (Fig. 1) with an ORF terminating at the same position as the one within the murine cDNA (data not shown). The longest ORF within the full-length human cDNA encodes a 949-amino-acid polypeptide that has a calculated molecular weight of 106,012. The 90-bp sequence preceding the first potential initiation codon (nucleotides 91 to 93) contains two in-frame stop codons. Since the nucleotide sequence around the next in-frame methionine (nucleotides 352 to 354) more closely resembles the Kozak consensus sequence for translation initiation (28), this residue was chosen as the initiation codon of a 862-amino-acid polypeptide (Fig. 1) with a calculated molecular weight of 95,797 that is in good agreement with the estimated molecular mass of DNA ligase III.

Alignment of peptides from bovine DNA ligase II and DNA ligase III within the ORF of the human DNA ligase III cDNA. Sequences homologous with the 20 peptides from bovine DNA ligase II and 16 of 18 peptides from bovine DNA ligase III (23, 60) have been identified within the ORF of the human DNA ligase III cDNA (Fig. 1). The degree of identity between the

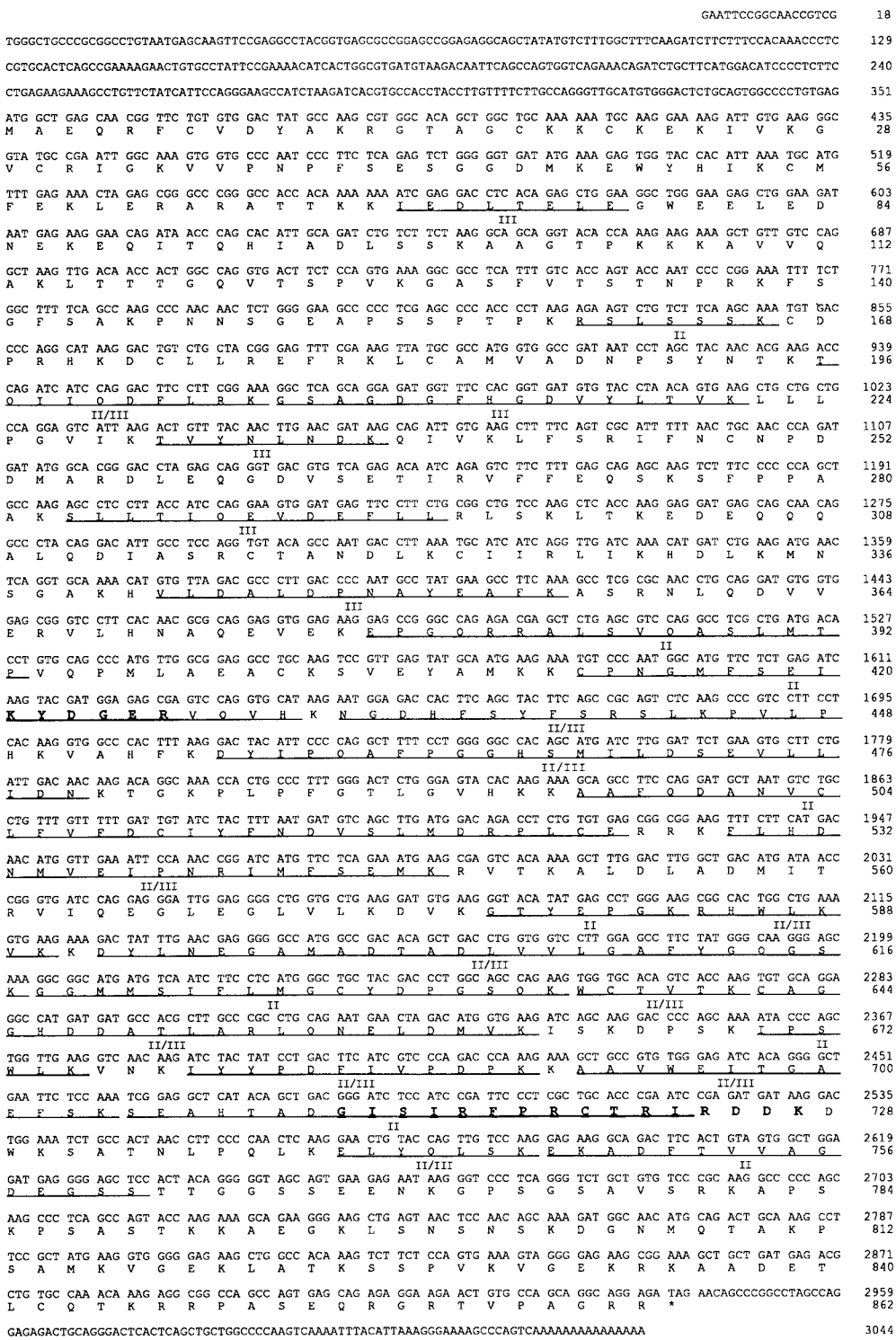

FIG. 1. Nucleotide sequence of human DNA ligase III cDNA. The predicted amino acid sequence is presented below the nucleotide sequence. Nucleotide and amino acid residues are numbered on the right. Regions homologous to the peptides from bovine DNA ligases II (indicated by II) and III (indicated by III) are underlined. Most of these peptides (16 of DNA ligase II and 13 of DNA ligase III) have been reported previously (23, 60). Four additional bovine DNA ligase II peptides homologous to regions in the human DNA ligase III ORF (TSLSSSK [160 to 166], EPGQRRALSVQASLMTP [377 to 393], ELYQLSK [740 to 746], EHAAFAITAGDEGSS [747 to 761]) and three additional bovine DNA ligase III peptides (IEDLTELE [70 to 77], SSAGDGFHGDVYLTVK [206 to 221], and ELYQLSK [740 to 746]) are also shown. Two bovine DNA ligase III peptides, SHLVAPCK and YFVAFDGDLV, could not be definitively aligned within the human DNA ligase III ORF. The putative active site of DNA ligase III and the conserved peptide present in eukaryotic DNA ligases are shown in boldface (4, 48, 57, 60).

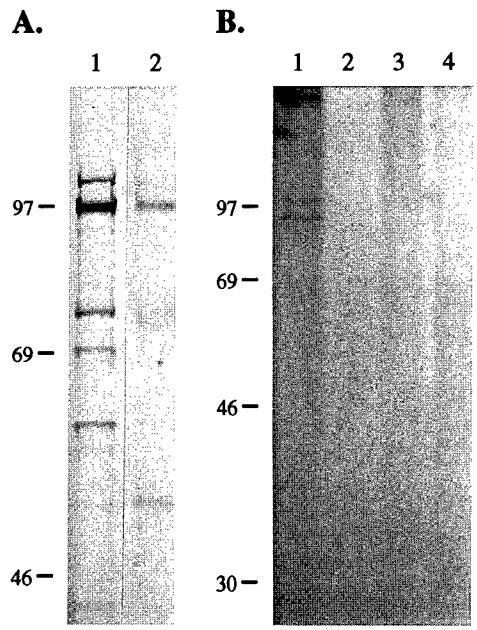

FIG. 2. In vitro translation of human DNA ligase III cDNA; reactivity of in vitro-translated DNA ligase III. (A) In vitro transcription and translation of full-length human DNA ligase III cDNA were performed as described in Materials and Methods. Lanes: 1, ^{35}S-labeled in vitro-translated polypeptides (2.5 μl); 2, polypeptides translated in vitro in the absence of labeled methionine purified by ammonium sulfate precipitation and adenylated by incubation with [α-^{32}P]ATP for 15 min at RT. Labeled polypeptides were separated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. The positions of ^{14}C-labeled molecular mass standards (Amersham) are indicated. (B) In vitro transcription and translation of human DNA ligase III cDNA with a 3′ deletion that removes the C-terminal 31 amino acids carried out in the absence of labeled methionine. After purification by ammonium sulfate precipitation, the reaction products were incubated with [α-^{32}P]ATP for 15 min at RT and then incubated for 2 h at 20°C with no addition (lane 1), 20 nmol of sodium PP_i (lane 2), 0.5 μg of oligo(dT)-poly(dA) (lane 3), and 0.5 μg of oligo(dT)-poly(rA) (lane 4).

bovine peptides and the predicted amino acid sequence of human DNA ligase III is greater than 90%. The human DNA ligase III cDNA encodes a sequence identical to the 16-amino-acid adenylylated peptide isolated from bovine DNA ligase II (60), indicating that Lys-421 is the active-site residue of DNA ligase III, and a sequence (residues 712 to 727) homologous with the conserved C-terminal peptide sequence present in eukaryotic DNA ligases (4, 48, 60).

On the basis of the alignment with human DNA ligase III, the bovine DNA ligase II peptides encompass about 66 kDa (Fig. 1). The 70-kDa DNA ligase II does not appear to be derived from DNA ligase III cDNA by initiating translation at an internal methionine, since there is no appropriately positioned methionine residue. In agreement with this prediction, incubation of polypeptides produced by in vitro translation of human DNA ligase III cDNA with [α-^{32}P]ATP did not produce a labeled 70-kDa polypeptide (Fig. 2A, lane 2). Thus, it appears probable that DNA ligases II and III are encoded by the same gene and are generated either by specific processing of a common polypeptide or by alternative splicing.

Human DNA ligase III cDNA encodes a catalytically active DNA ligase. Coupled in vitro transcription and translation of full-length human DNA ligase III cDNA (*LIG3*) in the presence of [^{35}S]methionine produced a major labeled band with a molecular mass of 97 kDa and a minor labeled band with a molecular mass of 106 kDa (Fig. 2A, lane 1). Similar reactions were performed in the absence of labeled methionine, and the translated products were assayed for DNA ligase activity. A polypeptide corresponding in size to the major translated product formed a labeled enzyme-adenylate complex (Fig. 2A, lane 2). After much longer exposure, a labeled 106-kDa enzyme-adenylate complex was also detected (data not shown). The 97- and 106-kDa polypeptides probably result from translation initiations at the methionine indicated in Fig. 1 and the first in-frame methionine (nucleotides 91 to 93 [Fig. 1]), respectively. The efficiency of translation and the reactivity of the 97-kDa polypeptide plus its similarity in size to purified DNA ligase III support the assignment of translation initiation shown in Fig. 1.

In vitro-translated DNA ligase III polypeptides lacking the C-terminal 31 amino acids did form labeled enzyme-adenylate complexes in similar assays (Fig. 2B, lane 1), whereas deletion of the C-terminal 160 amino acids inactivated the enzyme (data not shown). The labeled polypeptide-adenylate complexes were demonstrated to be authentic reaction intermediates by their reactivities with the polynucleotide substrates oligo(dT)-poly(dA) and oligo(dT)-poly(rA) (Fig. 2B, lanes 3 and 4). Similar results were obtained with 97-kDa DNA ligase III encoded by the full-length cDNA (data not shown). This utilization of oligo(dT)-poly(rA) as a substrate distinguishes DNA ligase III from DNA ligase I (55). Thus, the size and biochemical properties of the polypeptide encoded by human DNA ligase III cDNA are similar to those reported for purified bovine DNA ligase III (23, 55).

Homology of DNA ligase III with other DNA ligases of eukaryotes and eukaryotic viruses. As expected from the peptide sequencing data (23), DNA ligase III is highly related to the DNA ligase encoded by vaccinia virus (48). This homology, about 50% identity, extends over the entire translated sequence of vaccinia virus DNA ligase (Fig. 3). In contrast, the overall degree of identity shared between human DNA ligases I and III is only 17%. However, the putative catalytic domains of these enzymes exhibit about 30% identity, suggesting that the genes encoding DNA ligases I and III have evolved from a common ancestral gene. A comparison of DNA ligases encoded by eukaryotes and eukaryotic viruses indicates that there are two major families of DNA ligases (data not shown). The amino acid sequence homology appears to reflect conservation of function, since the majority of DNA ligases within the DNA ligase I family are known to be required for DNA replication. The other family of DNA ligases, which includes DNA ligase III and the DNA ligases encoded by the poxviruses, may have evolved to perform specific functions in eukaryotic DNA repair and/or genetic recombination.

The human DNA ligase III gene is located on chromosome 17. The chromosomal location of the DNA ligase III gene has been mapped by three different methods. An 810-bp fragment of DNA ligase III cDNA was used to probe genomic DNA from a BIOS somatic cell hybrid panel by Southern blotting. In the representative lanes shown in Fig. 4A, we demonstrate that although the human DNA ligase III probe cross-hybridizes with the hamster genomic DNA (Fig. 4A, lane 3), human DNA-specific bands were detected in somatic cell hybrids containing human chromosome 17 (Fig. 4A, lane 2) but not in somatic cell hybrids containing human chromosome 15 (Fig. 4A, lane 1), which contains the location of the BLM gene (32). Compilation of the data from the entire somatic cell hybrid panel (data not shown) demonstrated 100% concordance between the presence of human DNA-specific signals and human chromosome 17 and greater than 15% discordance for all other chromosomes.

Localization of the DNA ligase III gene to human chromosome 17 was independently confirmed by PCR analysis of rodent-human monochromosomal hybrids, including the

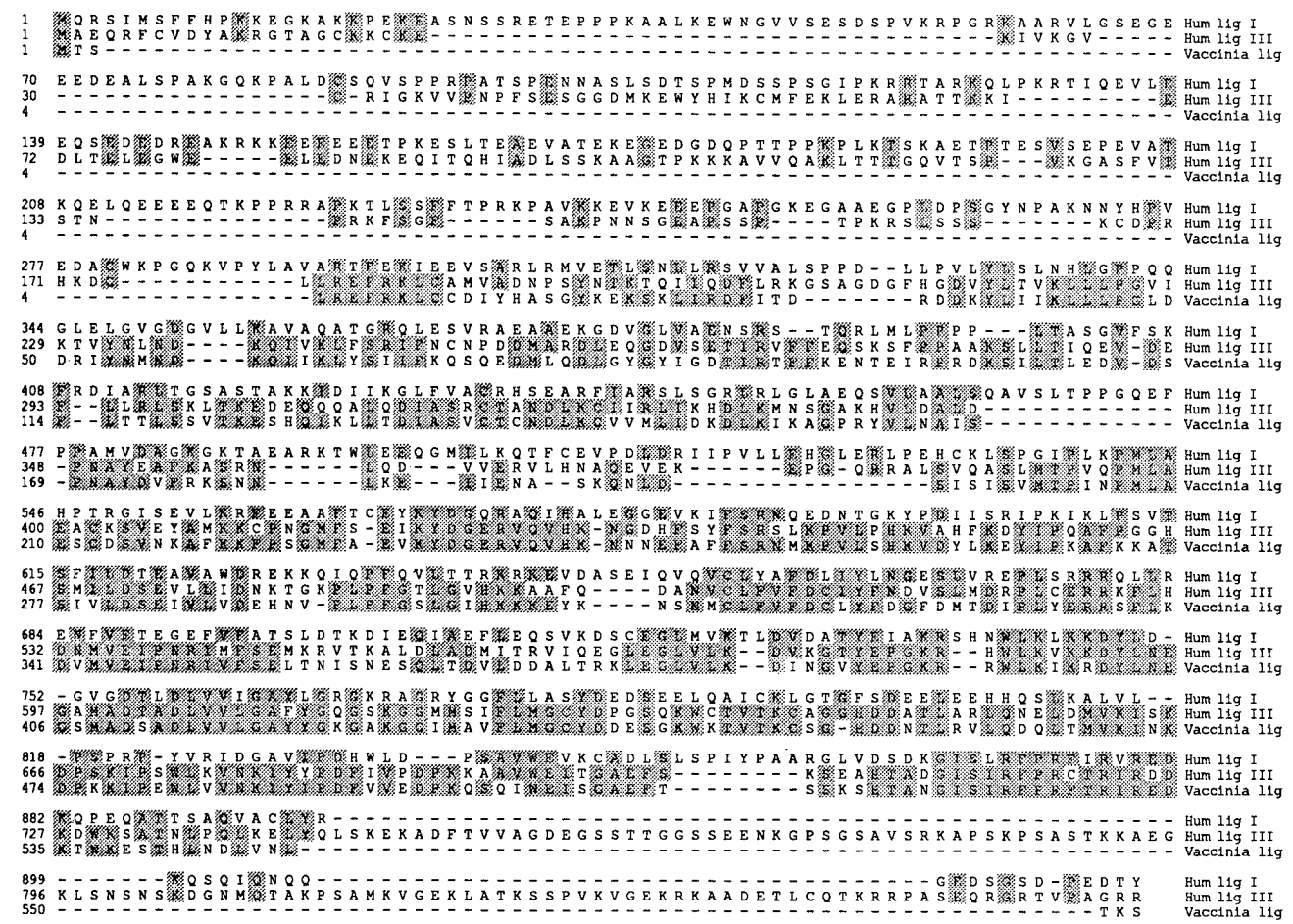

FIG. 3. Alignment of human DNA ligase (hum lig) I, human DNA ligase III, and vaccinia virus DNA ligase (vaccinia lig) protein sequences. The amino acid sequences of human DNA ligase I (4), vaccinia virus DNA ligase (48), and human DNA ligase III were aligned by using the DNAStar MegAlign program. Identical amino acids are indicated by shaded boxes. Gaps which have been introduced to maximize the alignment are indicated by dashes.

NIGMS Human-Rodent Somatic Cell Hybrid Mapping Panel 2. Primers specific to the 5′ region of the DNA ligase III cDNA produced an amplification product of approximately 850 bp with total human genomic DNA as the template (Fig. 4B). Amplification products were substantially smaller when mouse or hamster genomic DNA was used (Fig. 4B). The human DNA-specific product was amplified only in reactions with genomic DNAs from the mouse-human hybrid (A17), retaining only human chromosome 17 (32), and the somatic cell hybrid from the NIGMS panel containing only human chromosome 17 (Fig. 4B). Lastly, fluorescence in situ hybridization has been used to confirm the chromosome 17 localization of the DNA ligase III gene with the cDNA as a probe (data not shown).

DNA ligase III is highly expressed in testes. Expression of DNA ligase III in a variety of different human tissues and cells has been examined by Northern blotting with a 400-bp DNA ligase III cDNA fragment (nucleotides 2453 to 2843) as a probe. The probe hybridized to an mRNA species of about 3.6 kb in all tissues and cells examined except in testes, in which a doublet of 3.6- and 3.4-kb species was clearly visible with shorter exposures (see Fig. 5 and 7). Similar heterogeneity of mRNA transcripts in testes has been reported for cytochrome c_T (19) and lactate dehydrogenase (17) and appears to be due to different lengths of the poly(A) tail. The steady-state level of DNA ligase III mRNA in the liver was similar to that observed for the spleen. No transcripts of a different size that may encode DNA ligase II were detected (data not shown). The steady-state levels of DNA ligase III mRNA were significantly higher in testes than in any other tissue examined (Fig. 5). Quantitative analysis by scanning densitometry showed that the mRNA level in testes is at least 10-fold higher than those in other tissues and cells. Similar results were observed with a 5′ probe (nucleotides 1 to 538) (data not shown). For comparative purposes, we examined the expression of the replicative enzyme, DNA ligase I, in the same human tissues and cells. Testes also contain high steady-state levels of the 3.2-kb DNA ligase I mRNA. However, the highest levels of DNA ligase I mRNA were present in the thymus (Fig. 5).

Localization of DNA ligase III expression within mouse testes by in situ hybridization. Elevated levels of DNA ligase III mRNA (see above) and enzyme activity have been detected in mammalian testes (23), suggesting a role for this enzyme in spermatogenesis. To examine DNA ligase III expression in specific cell types within seminiferous tubules of adult mouse testes, we have isolated the murine cDNA homolog of DNA ligase III which exhibits about 85% homology at the nucleotide level with human DNA ligase III. The expression pattern of murine DNA ligase III mRNA was essentially the same as that observed for human DNA ligase III (Fig. 5) (data not shown). A murine DNA ligase III cDNA fragment, corresponding to nucleotides 296 to 960 of human DNA ligase III cDNA (Fig.

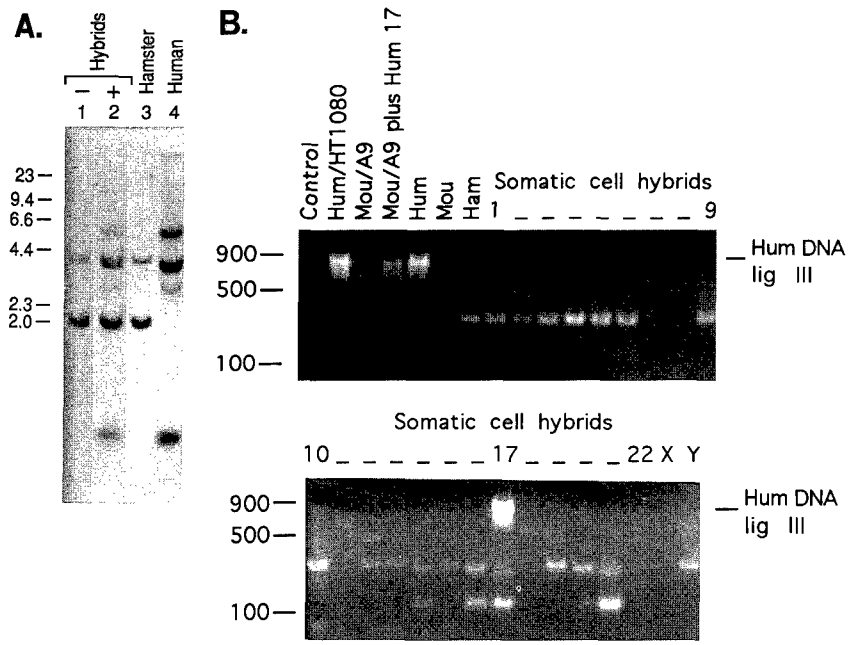

FIG. 4. Chromosomal localization of the DNA ligase III gene. (A) Southern analysis of *Pst*I-digested genomic DNAs (8 μg) from human, hamster, and hybrid somatic cells (BIOS Laboratory, Inc.) with an 810-bp *Eco*RI-*Xho*I DNA ligase III cDNA fragment as described in Materials and Methods. Only a selected panel of the hybridization results is shown. Lanes: 1, hamster cell line containing human chromosomes 4, 5, 7, 13, 15, 19, 21, and Y; 2, hamster cell line containing human chromosomes 8, 17, and 18; 3 and 4, hamster and human cell lines containing genomic DNA, respectively. The presence (+) or absence (−) of human chromosome 17 is indicated for each hybrid. The positions of size markers are shown on the left. (B) PCR amplification of a specific fragment of human (hum) DNA ligase (lig) III from monochromosomal somatic cell hybrids. Hum/HT 1080, human tumor cell line; Mou/A9, mouse cell line; Mou/A9 plus Hum 17, mouse cell line containing human chromosome 17; Hum, human parental DNA; Mou, mouse parental DNA; Ham, Chinese hamster parental DNA (parental DNA supplied with NIGMS Human Rodent Somatic Cell Hybrid Mapping Panel 2). 1 to 9, 10 to 22, X, and Y, individual human-rodent cell hybrids containing chromosomes 1 to 22 and X and Y, respectively. The 850-bp DNA ligase III fragment amplified from human genomic DNA and from somatic cell hybrids containing human chromosome 17 is indicated. The positions of molecular mass standards in base pairs are shown on the left.

1), was used to examine DNA ligase III expression in mouse testis sections by in situ hybridization. No hybridization was detected in experiments with the sense probe (Fig. 6A). In contrast, we observed significant hybridization in a small, discrete number of cells with the antisense probe (Fig. 6B). In similar experiments with antisense and sense probes from a 420-bp fragment of murine DNA ligase III cDNA corresponding to nucleotides 2034 to 2452 of human DNA ligase III cDNA, the same cell population hybridized with the antisense probe. On the basis of their morphological characteristics and their position within the seminiferous tubule, these cells were identified as primary spermatocytes. Thus, the high levels of DNA ligase III expression appears to be restricted to the stage of germ cell development when meiotic recombination occurs.

Expression of DNA ligases in developing mouse testes. Previously, we have shown that DNA ligases I and III are relatively highly expressed in adult testes compared with most other tissues and cells (Fig. 5). Since the temporal appearance of different cell types within testes and the cellular composition of testes at different days from birth to sexual maturity are well-documented (6), we have examined the steady-state mRNA levels of DNA ligases I and III in mouse testes as a function of age (Fig. 7). The highest levels of DNA ligase I expression were detected in the youngest animals (5 and 8 days old), with expression levels gradually declining with increasing age (Fig. 7a). This expression pattern correlates with the relative contribution of spermatogonia cells to the testes, ranging from 16 to 27% in the 5- and 8-day-old animals to 1% in adult animals (6). The high levels of DNA ligase I mRNA in the proliferating spermatogonia are consistent with the previously defined role of DNA ligase I in DNA replication (4, 5, 49, 58).

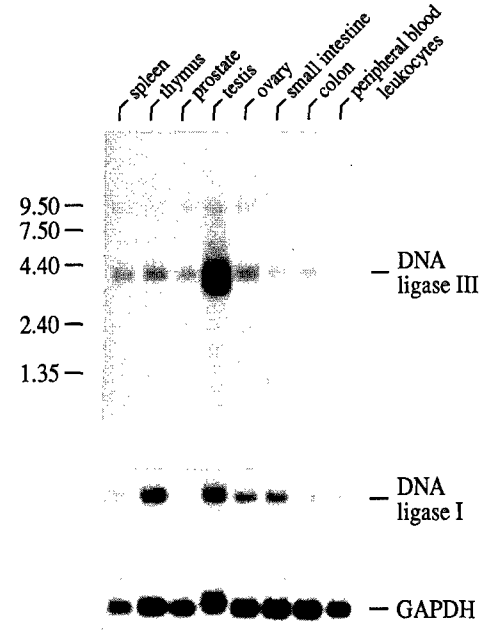

FIG. 5. Tissue and cell distribution of DNA ligase I and DNA ligase III mRNAs. A Northern blot containing ~2 μg of poly(A)$^+$ RNAs from various human tissues and cells was sequentially hybridized with a 400-bp human DNA ligase III cDNA fragment (nucleotides 2453 to 2843), a 1.4-kb DNA ligase I cDNA fragment (nucleotides 106 to 1544 [4]), and a 1.4-kb human GAPDH cDNA fragment as described in Materials and Methods.

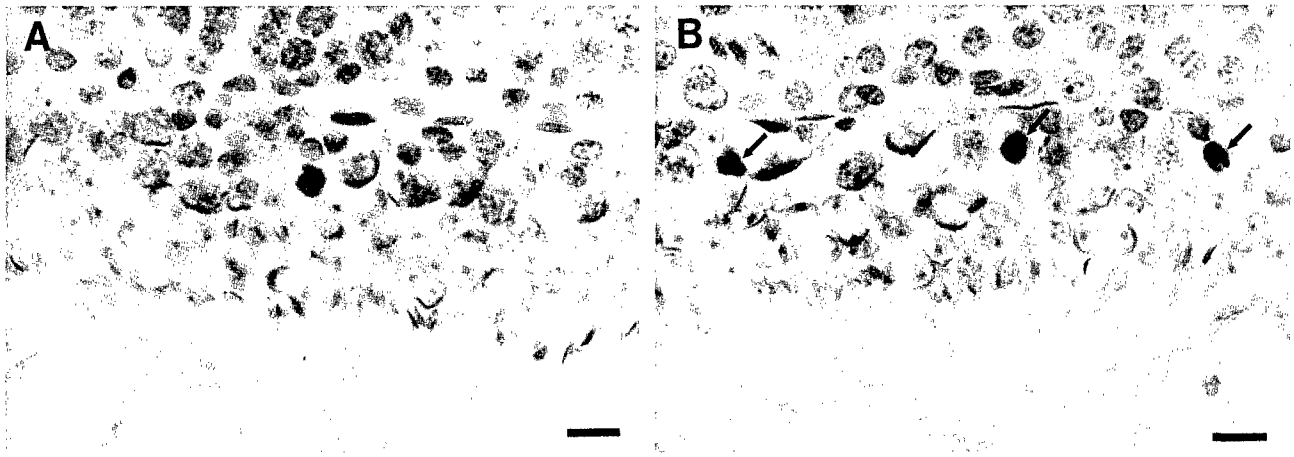

FIG. 6. Expression of DNA ligase III mRNA within the seminiferous tubules of mouse testes. In situ hybridization experiments were performed with mouse DNA ligase cDNA fragments as described in Materials and Methods. (A) Sense probe generated from the mouse cDNA fragment corresponding to nucleotides 296 to 960 of human DNA ligase III cDNA (Fig. 1); (B) antisense probe generated from the same fragment. The lumen of the seminiferous tubule is at the bottom of the photograph. Positively staining cells (pink color) are indicated by arrows. The scale is indicated by the bar (1 μm).

In contrast, DNA ligase III is expressed at low levels in the testes of mice that are as much as 15 days old. The highest levels of DNA ligase III steady-state mRNA were detected in the testes from 25-day-old mice, with about twofold lower levels in adult testes (Fig. 7b). In mice, primary pachytene spermatocytes first appear at approximately day 14 and constitute about 15% of the cells in the testes. Since it takes approximately 6 days to complete pachytene, cells representing the latter part of pachytene will not appear until approximately day 18 (6). At day 18, the relative contribution of pachytene spermatocytes to the testes reaches a peak of 36% and then gradually falls to 15% in adults. After day 18, the contribution of secondary spermatocytes to the testes remains constant at 1%, whereas round spermatids and condensing spermatids are present at significantly higher levels in adult testes. Thus, the expression of DNA ligase III mRNA correlates with the relative contribution of cells in the latter part of pachytene to the testes. This conclusion is compatible with the expression pattern observed by in situ hybridization (Fig. 6). The high levels of DNA ligase III expression in cells that are undergoing meiotic recombination suggest that this enzyme is required to seal DNA strand breaks that arise during recombination prior to meiotic division I.

DISCUSSION

We have described the cloning of a human cDNA that contains the complete ORF of DNA ligase III on the basis of the following observations: (i) 16 of the 18 peptide sequences obtained from purified bovine DNA ligase III (23) have been aligned within the amino acid sequence encoded by the cDNA (Fig. 1); (ii) in vitro transcription and translation of this cDNA produces a catalytically active DNA ligase that is similar in size and substrate specificity to bovine DNA ligase III (23, 55) (Fig. 2); and (iii) the predicted amino acid sequence of human DNA ligase III contains sequences that are highly homologous with the active-site motif (57, 60) and with a conserved C-terminal peptide sequence (4) that has been found in all ATP-dependent eukaryotic DNA ligases.

DNA ligases II and III were originally identified as minor activities in extracts from calf thymus glands (50, 55). Subsequent studies demonstrated that DNA ligase II is the major

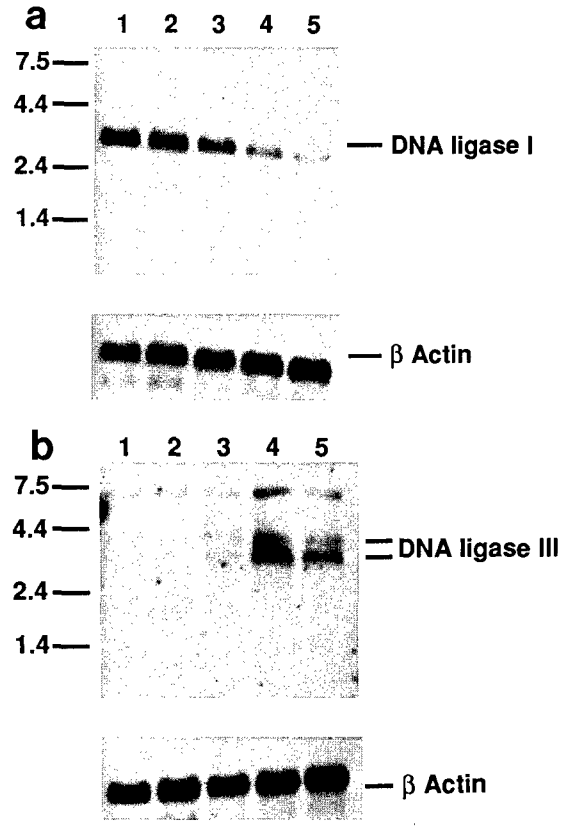

FIG. 7. Steady-state levels of DNA ligase I and DNA ligase III mRNAs in developing mouse testes. Duplicate poly(A)$^+$ samples (2 μg) from the testes of 5-day-old (lane 1), 8-day-old (lane 2), 15-day-old (lane 3), 25-day-old (lane 4), and 60-day-old (adult) (lane 5) mice were electrophoresed through 1.2% agarose-formaldehyde gel and then transferred to a nitrocellulose membrane as described in Materials and Methods. Prior to hybridization, the membrane was cut into two equivalent pieces. (a) The membrane sequentially hybridized with a murine DNA ligase I cDNA fragment (nucleotides 1734 to 2961 [46]) and a 2-kb human β-actin cDNA fragment; (b) the membrane sequentially hybridized with a murine DNA ligase III cDNA fragment (corresponding to nucleotides 1430 to 2452 of human DNA ligase III cDNA) and a 2-kb human β-actin cDNA fragment.

DNA joining activity in liver nuclei (60), whereas DNA ligase III is the major DNA joining activity in testis nuclei, with only trace amounts of DNA ligase II detected (23). Amino acid sequencing of peptides from bovine DNA ligases II and III indicated that these enzymes may be encoded by the same gene (23, 60). In agreement with this hypothesis, we have been able to align all of the DNA ligase II and the majority of the DNA ligase III peptides within the ORF of human DNA ligase III. In addition, we have not detected any highly homologous but distinct DNA sequences in 16 independent cDNA clones that hybridized with the PCR probes. In Northern blotting experiments, a single 3.6-kb RNA species was detected at a low level in all tissues (including the liver) except for testis tissue (Fig. 5). In testes, significantly higher levels of the 3.6-kb mRNA and also a 3.4-kb mRNA were observed. Thus, the expression patterns of mRNAs that hybridized with DNA ligase III probes do not indicate that DNA ligases II and III are generated by alternative splicing.

An alternative possibility is that DNA ligase II is derived from DNA ligase III by the action of endogenous proteases during protein purification. However, we have not observed the appearance of a catalytically active 70-kDa polypeptide in partially purified fractions of DNA ligase III. Furthermore, the addition of proteases to DNA ligase III fractions also failed to produce an active fragment similar in size to DNA ligase II (40, 55). The amino terminus of the 70-kDa DNA ligase II was blocked to Edman degradation (60), suggesting that the amino-terminal residue is modified, as is the case with the primary translation product of most soluble proteins (7). It seems unlikely that DNA ligase II is created from DNA ligase III by a C-terminal deletion, since this would remove an essential portion of the catalytic domain. Thus, these polypeptides may be related by a specific proteolytic processing pathway that involves modification of the amino-terminal residue and/or protein splicing (13).

A comparison of human DNA ligase III with ATP-dependent DNA ligases encoded by eukaryotes and eukaryotic viruses suggests that DNA ligase III has the same domain structure as these enzymes, consisting of a catalytic C-terminal domain and a nonessential amino-terminal domain that may be the site of posttranslational modifications and protein-protein interactions (4, 37, 57). Analysis of the evolutionary relationship between these enzymes suggests that there are two families of eukaryotic DNA ligases. There is considerable genetic and biochemical evidence demonstrating that members of the DNA ligase I family are required for DNA replication (4, 26, 33, 34, 49, 58). Within the other family, DNA ligase III is most highly related to the DNA ligase encoded by vaccinia virus, the prototypic poxvirus. Since the only effect on viral DNA metabolism of deleting the DNA ligase gene is to increase the sensitivity of the mutant virus to treatment with DNA-damaging agents, it appears that the viral enzyme functions in DNA repair (12, 27). DNA ligase III is also involved in DNA repair (8) and recombination in somatic cells (24). The studies reported here indicate that DNA ligase III may play a specific role during meiotic recombination. Therefore, this second family of DNA ligases appears to function in specific DNA repair and genetic recombination pathways.

The phenotypes of cell lines established from patients with the hereditary cancer-prone disease BLM are consistent with a defect in DNA joining (30, 41). Furthermore, it has been reported that BLM cell extracts contain altered high-molecular-weight DNA ligase activity (10, 61, 62). The absence of mutations in the BLM DNA ligase I (5, 36) and the localization of the DNA ligase I gene to chromosome 19 (4) and the BLM gene to chromosome 15 (32) eliminated DNA ligase I as a candidate gene for BLM. The association of DNA ligase III with the product of the human DNA repair gene *XRCC1* (8) made DNA ligase III an attractive candidate for BLM, since the hallmark cytogenetic feature of both BLM cell lines and the mutant *xrcc1* cell line EM9 is a spontaneously elevated frequency of sister chromatid exchange (9, 15). Although the BLM and *XRCC1* genes are located on different chromosomes (32, 47), mutations in either gene would be expected to produce similar phenotypes if the gene products function in the same pathway. The localization of the DNA ligase III gene to chromosome 17 (Fig. 4) eliminates this gene as a candidate for BLM and is consistent with the presence of wild-type levels of DNA ligase III activity in BLM cells (56). Therefore, the abnormal DNA ligation associated with BLM may be caused by mutations in a gene that functions in the same pathway as DNA ligase III and Xrcc1 proteins or that regulates DNA ligase activity.

The steady-state mRNA levels of both DNA ligase I and DNA ligase III correlate with measurements of enzyme activity in different tissues. For DNA ligase I, the highest levels of enzyme activity (50) and mRNA are found in the thymus, with relatively high levels of DNA ligase I mRNA also detected in the testes (Fig. 5). In addition to being required for DNA replication, DNA ligase I may perform a specific function in the immune system, since the patient with inherited DNA ligase I mutations presented with severe combined immunodeficiency (5). During the development of haploid gametes, diploid germ cells undergo a cycle of DNA replication without cell division. In the yeast *Saccharomyces cerevisiae*, expression of the *CDC9* gene, which is functionally homologous to mammalian DNA ligase I, is induced during premeiotic DNA synthesis and then rapidly declines (25). During mammalian spermatogenesis, the highest levels of DNA ligase I mRNA are found in the proliferating spermatogonia. This is consistent with the previous observation that DNA ligase I activity is significantly higher in prepubertal spermatogonia than in meiotic spermatocytes and later cell types (21). Therefore, DNA ligase I appears to fulfill the same function, i.e., joining Okazaki fragments, during premeiotic DNA synthesis that it does during mitotic DNA replication (58).

In the case of DNA ligase III, the highest levels of mRNA (Fig. 5) and enzyme activity (23) are found in the testes. This elevated expression has been localized to primary spermatocytes by in situ hybridization (Fig. 6). To confirm this observation, DNA ligase III expression was also examined in the developing testes. These studies indicated that the elevated levels of DNA ligase III mRNA occur in the latter part of pachytene prior to meiotic division I. A similar expression pattern has been observed for the DNA repair gene *XRCC1* (59), whose product interacts with DNA ligase III (8). During pachytene, homologous chromosomes align within the synaptonemal complex and genetic exchanges take place (18, 35, 52, 53). Thus, the temporal expression of DNA ligase III during germ cell development suggests that this enzyme functions together with Xrcc1 to seal breaks in DNA that have arisen as a consequence of meiotic recombination events. In somatic cells, DNA ligase III presumably functions as a DNA repair enzyme, sealing single-strand breaks generated as a consequence of DNA damage. The availability of DNA ligase III cDNA should facilitate investigation of the mechanisms of eukaryotic DNA repair and genetic recombination, in particular meiotic recombination.

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J.W.C., A.E.T., and I.H. contributed equally to this work.

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The University of Texas
Health Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, Texas 78284-7822

Institutional Animal Care Program

(210) 567-3718 **FAX (210) 567-3708**

November 1, 1996

U.S. Army Medical Research And Development Command

Dear Sir or Madam:

The following protocol was reviewed and approved by The Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio:

TITLE: Training Program In The Molecular Basis Of Breast Cancer

PRINCIPAL INVESTIGATOR: Wen-Hwa Lee, Ph.D.

PROTOCOL NUMBER: 94062-99-04-U*

APPROVAL DATE: November 1, 1996

This Institution has an Animal Welfare Assurance on file with the Office of Protection from Research Risks. The Assurance Number is A3345-01.

Sincerely,

Steven W. Mifflin, Ph.D., Chairman
Institutional Animal Care and Use Committee

*Umbrella identification number; no animals may be ordered under this number.
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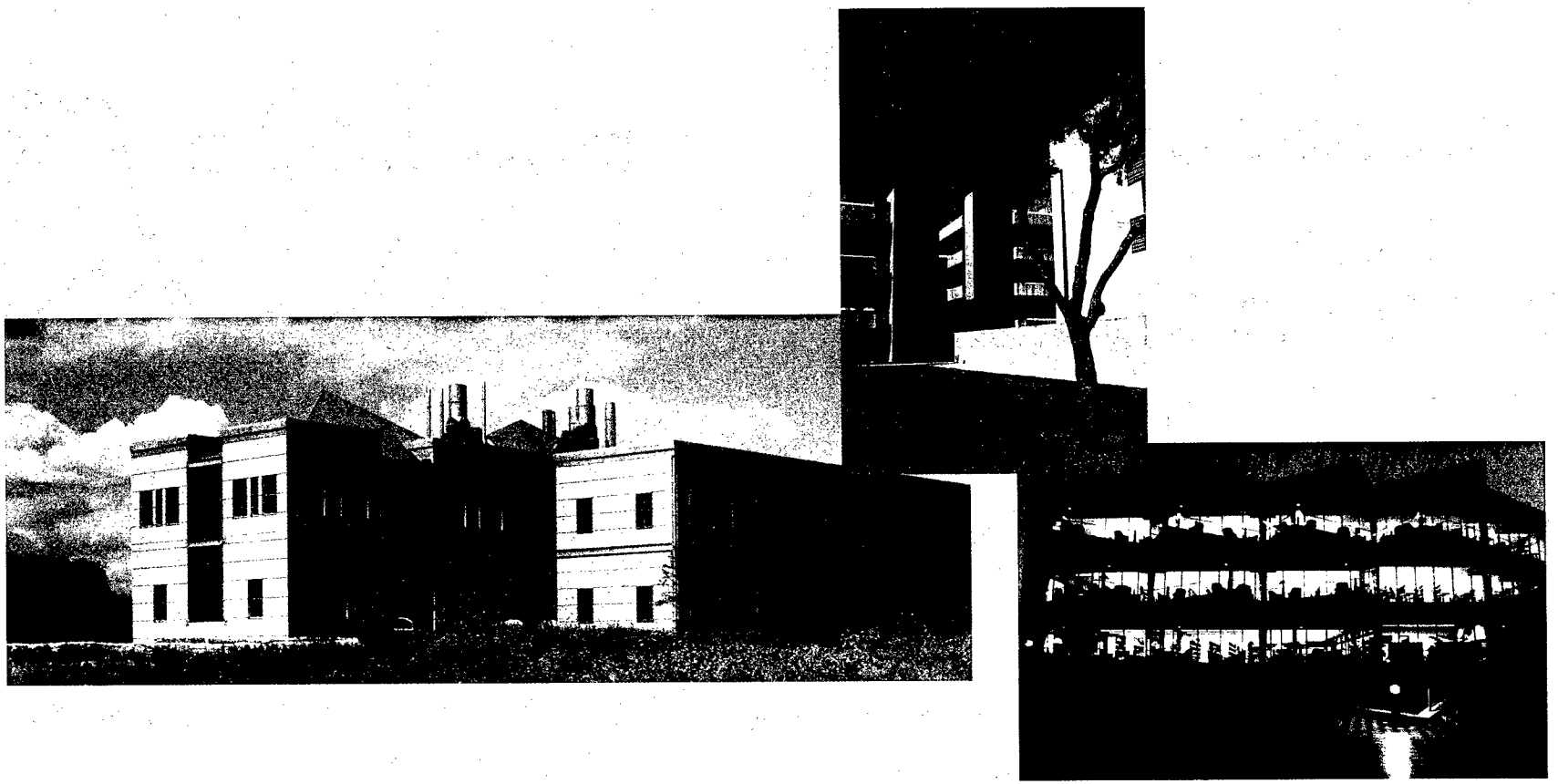


The Graduate Program In *Molecular Medicine*

A Message from the Director

The Graduate Program in Molecular Medicine was founded on the understanding that basic research in molecular and cellular biology is fundamentally important to the advancement of the medical sciences. The faculty of the Molecular Medicine Program is, by design, interdisciplinary and interdepartmental, including both basic scientists and physician scientists. It consists of nationally recognized experts in the fields of cancer biology, development, and neurobiology affording students a wide range of research projects from which to choose.

At the heart of the Program is the close participation of basic and clinical scientists in every step of the Ph.D. educational process. A goal of the Program is to train tomorrow's scientists to be better prepared to direct their basic molecular and cellular research toward issues of medical relevance. Importantly, Molecular Medicine trainees will also be more attuned to the biomedical sciences which will facilitate their recognition of clinically relevant findings arising through serendipity.

After two semesters, including the successful completion of core course requirements in Advanced Molecular and Cellular Biology and Molecular Medicine, and passing comprehensive examinations, students choose advisors from the Molecular Medicine faculty. The scientists in the Program direct their research projects in laboratories located on the campuses of The University of Texas Health Science Center at San Antonio and The University of Texas Institute of Biotechnology. Each student selects a dissertation advisory committee from the faculty that must be composed of both clinical and basic scientists. Interdisciplinary approaches to the solution of basic research problems are realized by this approach.

Wen-Hwa Lee, Ph.D.

Table of Contents

Meet the Faculty Researchers

Students in the Program are supported by beginning stipends of $15,000 from institutional sources, individual research and Program training grants. Outstanding students are offered special Kozmetsky Fellowships in Molecular Medicine. The qualifications for acceptance into the Program include a GPA exceeding 3.00, combined verbal and quantitative GRE scores of 1000 and outstanding letters of reference. Students wishing additional information should contact the Applicant Advisor, Graduate Program in Molecular Medicine, The University of Texas Institute of Biotechnology, 15355 Lambda Drive, San Antonio, Texas 78245. 210-567-7251 is for information by telephone and gardner@uthscsa.edu is for e-mail inquiries.

Hanna E. Abboud, M.D.

Professor, Department of Medicine
M.D., Alexandria University of Egypt

Research efforts in this laboratory focus on the role of cytokines in progressive renal disease. The biologic effects of purified recombinent cytokines in cultured renal cells are characterized including chemotaxis, DNA synthesis, and cell contraction. In addition, the signal transduction mechanisms, that mediate the effect of these cytokines, including membrane, cytoplasmic, and nuclear factors, are explored. The applicability of the *in vitro* findings is explored *in vivo* in models of renal disease that involve cell proliferation or hypertrophy. The *in vivo* studies examine the changes in the expression and activity (up or down regulation) of signal transduction proteins in isolated nephron segments. The precise role of the signal transducing proteins in the functional or morphologic injury is explored using inhibitors, antisense oligonucleotides, or gene targeting. Techniques used to achieve these goals include cell culture, *in vivo* animal studies, immunohistochemistry and *in situ* hybridization, immunoprecipitation, Northern blotting, solution hybridization analysis, DNAase footprinting, gel retardation, Southwestern blotting, screening of libraries, and establishment of transgenic animals.

Representative Publications:

1. Silver, B.J., Jaffer, F.E., Abboud H.E. PDGF synthesis in human mesangial cells: Induction by multiple peptide mitogens. Proc. Natl. Acad. Sci. U.S.A. 86:1056-1060, 1989.

2. Knauss, T.C., Jaffer, F., Abboud, H.E. Phosphatidic acid modulates DNA synthesis, phospholipase C, and PDGF mRNAs in mesangial cells. J. Bio. Chem. 265:14457-14463, 1990.

3. Barnes, J.L., Abboud, H.E. Temporal expression of autocrine growth factors corresponds to morphologic features of mesangial proliferation in habu snake venom-induced glomerulonephritis. Am. J. Pathology 143:1366-1376, 1993.

4. Abboud, H.E., Pinzani, M., Knauss, T., Pierce, G.F., Hsieh, P., Kenney, W., Jaffer. Actions of platelet-derived growth factor isoforms in mesangial cells. J. Cell Physiol. 158:140-150, 1994.

5. Wenzel, U., Fouqueray, B., Biswas, P., Grandaliano, G., Ghosh Choudhury, G., and Abboud, H.E. Activation of mesangial cells by phosphatase inhibitor vanadate: Potential implications for diabetic nephropathy. J. Clin. Invest., In Press 1995.

S. Atherton, Ph.D.

Professor, Cellular & Structural Biology
Ph.D., The University of Texas Health Science Center
at San Antonio

Research Interests: My laboratory is interested in the pathogenesis of ocular diseases caused by members of the herpesvirus family. We are studying the virologic and immunologic mechanisms of herpes simplex virus type 1 (HSV-1) retinitis by examining the ocular-central nervous system axis and the role that T cells and other effector modalities such as cytokines play in determining patterns of virus infection in the eyes and central nervous system. We are also using a murine model of cytomegalovirus (CMV) retinitis (the most common infectious ocular complication of the acquired immunodeficiency syndrome) to gain insight into mechanisms of human CMV infection during immunosuppression. For this project, we use a variety of immunologic, molecular, and histologic techniques to determine sites of acute and latent infection, to study immunologic and non-immunologic factors which control reactivation from latency, and define strategies to protect the retina from viral invasion.

Selected Publications

Atherton, S.S. Acute retinal necrosis (ARN): What we know from the mouse. In Infectious Diseases of the Eye. pp. 428-436, Bialasiewicz, A.A. and Schaal, K.P., eds. Æolus Press, Buren, The Netherlands, 1994.

Azumi, A., Cousins, S.W., Kanter, M.Y., and Atherton, S.S. Modulation of murine HSV-1 retinitis in the uninoculated eye by CD4+ lymphocytes. Invest. Ophthalmol. Vis. Sci. 35:54-63, 1994.

Duan, Y., Ji, Z., and Atherton, S.S. Dissemination and replication of murine cytomegalovirus after supraciliary inoculation in immunosuppressed mice. Invest. Ophthalmol. Vis. Sci. 35:1124-1131, 1994.

Azumi, A. and Atherton, S.S. Sparing of the ipsilateral retina following anterior chamber inoculation of HSV-1: Requirement for either CD4+ or CD8+ T cells. Invest. Ophthalmol. Vis. Sci. 35:3251-3259, 1994.

Zhao, M., Azumi, A., and Atherton, S.S. T lymphocyte infiltration in the brain following anterior chamber inoculation of HSV-1. J. Neuroimmunol. 58:11-19, 1995.

David H. Boldt, M.D.

Professor, Department of Medicine
M.D., Tufts University School of Medicine

Our laboratory demonstrated that two eukaryotic cell proteins, protein kinase C-ß and tartrate-resistant acid phosphatase (TRAP), are subject to a complex mechanism of iron-responsive transcriptional regulation. Both genes are transcriptionally active in the presence of ferric transferrin (FeTF), whereas both are inhibited at the transcriptional level in the presence of exogenous hemin. In each case, the 5'-flanking DNA contains sequences responsive to FeTF or hemin. TRAP is an iron-containing protein encoded by the same gene encoding uteroferrin, a placental iron transport protein. Current research uses the mouse (M)-TRAP gene as a model to elucidate molecular mechanisms of hemin-responsive transcriptional regulation. The mTRAP promoter has 2 nonoverlapping regions with promoter activity. The upstream promoter is transcriptionally regulated by FeTF and hemin. The mTRAP 5'-flanking region has been sequenced to -1846 bp relative to ATG and contains no classic stem loop iron response elements (IREs). However, we identified a hemin-responsive nuclear transcription factor (HRP) which binds to a 27-bp sequence at -1815 to -1789 bp relative to ATG. A tandem repeat sequence, GAGGC;GAGGC, contained within this DNA segment has been shown to be critical for binding of HRP. Binding of HRP is inhibited by anti-HAP1 antibodies indicating homology between the hemin-dependent factor and the yeast heme-dependent transcription factor, HAP1. Research in this area now encompasses the following aims: (1) to identify, clone, and sequence the gene(s) encoding HRP from human U937 cell expression library; (2) to characterize interactions of HRP with DNA; (3) to investigate whether interactions of HRP with other transcription regulatory elements are required for its inhibitory effect on mTRAP expression; (4) to express cloned factors in bacterial and yeast expression systems to facilitate structure/function studies; and (5) to test whether other mammalian genes with noncoding GAGGC/GAGGC motifs are similarly regulated by hemin. We plan to use PCR differential hybridization screening and/or differential display PCR to identify new genes whose expression is regulated by iron or hemin and to examine the roles of these gene products in hematopoietic cell proliferation and differentiation.

Selected Publications

Alcantara, O., Javors, M. and Boldt, D.H. Induction of protein kinase C mRNA in cultured lymphoblastoid T cells by iron transferrin but not by soluble iron. Blood 77:1290-1297, 1991.

Alcantara, O., Obeid, L., Hannun, Y., Ponka, P. and Boldt, D.H. Regulation of protein kinase C (PKC) expression by iron: Effect of different iron compounds on PKC-ß and PKC-a gene expression and role of the 5'-flanking region of the PKC-ß gene in the response to ferric transferrin. Blood 84:3510-3517, 1994.

Alcantara, O., Reddy, S.V., Roodman, G.D. and Boldt, D.H. Transcriptional regulation of the tartrate-resistant acid phosphatase (TRAP) gene by iron. Biochem. J. 298:421-425, 1994.

Reddy, S.V., Hundley, J.E., Windle, J.J., Alcantara, O., Leach, R.J., Boldt, D.H. and Roodman, G.D. Characterization of the mouse tartrate-resistant acid phosphatase (TRAP) gene promoter. J. Bone Min. Res., in press.

Reddy, S.V., Alcantara, O., Roodman, G.D. and Boldt, D.H. Inhibition of tartrate-resistant acid phosphatase (TRAP) gene expression by hemin. Identification of a hemin responsive inhibitor of transcription. Submitted for publication.

Barbara H. Bowman, Ph.D.

Professor And Chair, Department of Cellular and Structural Biology
Ph.D., The University of Texas at Austin

Dr. Bowman studies gene expression and expression of genes leading to age-associated diseases in humans. She uses transgenic mice carrying specific genes related to age-related diseases and the aging process. Gene knockouts are being initiated so that only specific human genes will be expressed in the developing and aging mouse. This will be especially useful in studying two genes encoding the amyloid precursor protein and the apolipoprotein E4 protein. These two genes are expressed in brains of humans with Alzheimer's disease and have been strongly associated with the higher risk of developing Alzheimer's disease. The model of transgenic mice that carry and overexpress only the human amyloid precursor protein and the apolipoprotein E4 genes (without the mouse gene equivalents) will offer opportunities to analyze mutations that may intensify or remove the threat of developing the pathology and memory loss associated with Alzheimer's disease. This is a multidisciplinary project with investigators having expertise in neuropathology, rodent memory, genetics and transgenic and knockout mice.

Selected Publications

Yang, F., Jansen, L., Friedrichs, W.E., Buchanan, J.M. and Bowman, B.H. IL-1b decreases expression of amyloid precursor protein gene in human glioma cells. Biochem. Biophys. Res. Comm. 191:1014-1019, 1993.

Lu, Y., Bowman, B.H. and Adrian, G.S. The postnatal onset of expression of chimeric human transferrin-chloramphenicol acetyl-transferase genes in transgenic mouse liver involves hepatic nuclear factors. Submitted, 1995.

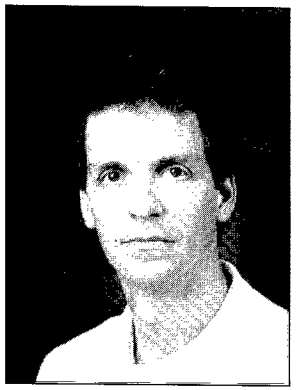
Martinez, A., Jansen, L., Buchanan, J.M., Adrian, G.S., Yang, F., Herbert, D.C., Weaker, F.J., Walter, C.A. and Bowman, B.H. A human (3.3 kb) haptoglobin-CAT transgene is modulated in lungs of transgenic mice by inflammation. Biochem. Biophys. Res. Comm. 208:309-315, 1995.

Friedrichs, W.E., Navarijo-Ashbaugh, A.L., Bowman, B.H. and Yang, F. Expression and inflammatory regulation of haptoglobin gene in adipocytes. Biochem. Biophys. Res. Comm. 209:250-256, 1995.

Bowman, B.H., Jansen, L., Yang, F., Adrian, G.S., Zhao, M., Atherton, S.S., Buchanan, J.M., Greene, R., Walter, C., Herbert, D.C., Weaker, F.J., Chiodo, L.K., Kagan-Hallet, K. and Hixson, J.E. Discovery of a brain promoter from the human transferrin gene and its utilization for development of transgenic mice that express human APOE alleles. Proc. Natl. Acad. Sci., in press, 1995.

Steven G. Britt, M.D.

Assistant Professor, Institute of Biotechnology
M.D., The University of Texas Medical Branch
at Galveston



Color vision is an excellent example of how neurons are tuned to different stimuli. This process requires both a developmental program to recruit cells to different fates, as well as different receptor molecules that will provide the mature differentiated cells with unique sensitivities. Our ability to see in color is based upon the execution of such a program, that produces three different classes of neuronal cone photoreceptor cells in the retina. These cells express different forms of the "light receptor" rhodopsin, that are sensitive to either red, blue, or green light.

I am interested in studying the structural features of the different rhodopsins that confer differences in spectral sensitivity, and in examining the developmental steps involved in specifying their expression. In order to examine these biological problems, we have used the fruit fly (*Drosophila melanogaster*) as an experimental animal.

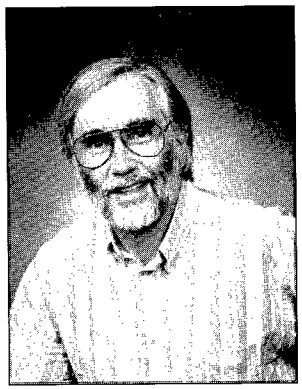
Flies discriminate between colors in the same way that humans do, and have rhodopsins that are sensitive to blue, purple, green, and ultraviolet light. In our experiments, we have taken blind mutant flies which lack the normal light receptor, and reintroduced genes which encode hybrid rhodopsins. These hybrid molecules were genetically engineered and are composed of different regions of the purple and blue light receptors. The mutant flies which express these hybrid rhodopsins are now responsive to light. We measured the electrical response of their eyes to different colors of light, and were able to identify regions of the light receptor protein which are involved in color tuning. Using these and other techniques, we are also studying the development of the eye and the genes that determine which form of rhodopsin is expressed in a specific photoreceptor cell. In addition, we are studying how mutations in rhodopsin may be involved in degenerative retinal diseases in humans, such as retinitis pigmentosa.

Selected Publications

Britt, S.G., Feiler, R., Kirschfeld, K. and Zuker, C.S. Spectral tuning of rhodopsin and metarhodopsin *in vivo*. Neuron, 11:29-39, 1993.

John M. Chirgwin, Ph.D.

Associate Professor, Department of Medicine
Ph.D., University of California, Riverside



The laboratory is broadly interested in the cellular sorting of nascent proteins, in particular to the lysosome and into nonclassical secretory pathways. At the bench the experiments generally involve making mutants (guided by X-ray crystallographic structures) which are predicted to affect the sorting steps taken by the proteins. Mutants are made by PCR and expressed in both mammalian cells and in *E. coli*; the former to test sorting and the latter to assess structure and folding of the mutant proteins. Stably miss-sorted mutants are also used to identify cellular binding proteins by chemical crosslinking.

Most secreted proteins pass through the endoplasmic reticulum and Golgi, through secretory vesicles, and out of the cell. However, a number of proteins are exported from the cytoplasm via nonclassical pathways. Rhodanese, normally a mitochondrial enzyme (1), is exported by such a pathway when expressed without its N-terminal mitochondrial import signal (2). The protein enters a high molecular weight complex in the cytoplasm which keeps the protein unfolded. This complex may then extrude the unfolded rhodanese through a pore in the plasma membrane to the outside medium. We are attempting: (1) to purify the cytoplasmic complex; (2) to determine why rhodanese mutants enter this complex; and (3) to identify the membrane pore by chemical crosslinking.

As lysosomal procathepsin D is synthesized through the endoplasmic reticulum and golgi, it is modified by the addition of mannose 6-phosphate (3,4). The protein then binds to intracellular mannose 6-P glycoprotein receptors which deliver it to the forming lysosome. Related proteins, such as pepsinogen, do not receive mannose 6-P modification. Recent experiments in our laboratory indicate that the signal for addition of mannose 6-P is carried in the 44 amino acid pro sequence of procathepsin D (5). We are currently mutating specific regions of this sequence to define the signal.

Selected Publications

Miller-Martini, D.M., Chirgwin, J.M. and Horowitz, P.M. Mutations of noncatalytic sulfhydryl groups influence the stability, folding, and oxidative susceptibility of rhodanese. J. Biol. Chem. 269: 3423-3428, 1994.

Sloan, I.S., Horowitz, P.M. and Chirgwin, J.M. Rapid secretion by a nonclassical pathway of overexpressed mammalian mitochondrial rhodanese. J. Biol. Chem. 269:27625-27630, 1994.

Schorey, J.S., Fortenberry, S.C. and Chirgwin, J.M. Lysine residues in the C-Terminal lobe and lysosomal targeting of procathepsin D. J. Cell. Sci. 108:2007-2015, 1995.

Fortenberry, S.C., Schorey, J.S. and Chirgwin, J.M. Role of glycosylation in the expression of human procathepsin D. J. Cell. Sci. 108:2001-2006, 1995.

Fortenberry, S.C. and Chirgwin, J.M. The propeptide is nonessential for the expression of cathepsin D. J. Biol. Chem. 270:9778-9782, 1995.

Barbara A. Christy, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., The Johns Hopkins University

My laboratory is studying regulatory proteins involved in control of growth and differentiation in mammalian cells and in developing embryos. We previously identified several growth factor-regulated genes that encode transcriptional regulatory proteins which affect expression of other genes. One gene (Zif268) encodes a protein with a zinc finger DNA-binding domain. Zif268 protein binds to DNA and affects transcription of a number of genes. Zif268 can up-regulate or down-regulate transcription depending on the context of its binding sites. In addition, we believe that Zif268 protein is controlling different target genes in different cell types. To define the mechanism by which the Zif268 protein acts to affect transcription, we are attempting to identify proteins which interact with Zif268 protein and target genes that are regulated by Zif268.

Another project focuses on the role of two related helix-loop-helix (HLH) proteins, Id3 and Id4. HLH proteins have been implicated in the control of differentiation and development, and they share a conserved HLH motif that is important for protein-protein interactions between HLH family members. The Id class of HLH proteins lacks a DNA-binding domain and are thought to act as dominant negative regulators of other proteins. In cultured muscle cells, we find that Id3 is down-regulated upon differentiation and ectopic expression of Id3 inhibits differentiation. In mouse embryos, Id3 mRNA expression is most abundant in the neuroepithelial cells of the brain, in somites and developing muscle, bone and cartilage. Id4 mRNA expression is more restricted, limited to the developing brain, spinal cord and the neurohypophesis. Since Id4 expression appears to be restricted to neuronal cells, we are testing the ability of ectopic Id4 to affect neuronal cell differentiation. Since these Id proteins may be acting as negative regulators of other HLH proteins, we are screening to identify proteins which interact with the two Id proteins, and have identified several candidates. The long-term goal of our laboratory is to define the role of these and related proteins in the control of growth and differentiation.

Selected Publications

Christy, B.A., Sanders, L.K., Lau, L.F., Copeland, N.G., Jenkins, N.A. and Nathans, D. An Id-related Helix-Loop-Helix Protein Encoded by a Growth Factor-Inducible Gene. Proc. Natl. Acad. Sci. USA 88:1815-1819, 1991.

Worley, P.F., Christy, B.A., Nakabeppu, Y., Bhat, R.V., Cole, A.J. and Baraban, J.M. Constitutive Expression of Zif268 in Neocortex is Regulated by Synaptic Activity. Proc. Natl. Acad. Sci. USA 88:5106-5110:1991.

Murphy, T.H., Worley, P.F., Nakabeppu, Y., Christy, B., Gastel, J. and Baraban, J.M. Synaptic Regulation of Immediate Early Gene Expression in Primary Cultures of Cortical Neurons. J. Neurochem. 57:1862-1872, 1991.

Nikam, S.S., Tennekoon, G.I., Christy, B.A., Yoshino, J.E. and Rutkowski, J.L. The Zinc Finger Transcription Factor Zif268/Egr-1 is Essential for Schwann Cell Expression of the p75 NGF Receptor. Neuron, in press, 1995.

Melnikova, I.M. and Christy, B.A. Muscle Cell Differentiation is Inhibited by the Helix-Loop-Helix Protein Id3. Submitted, 1995.

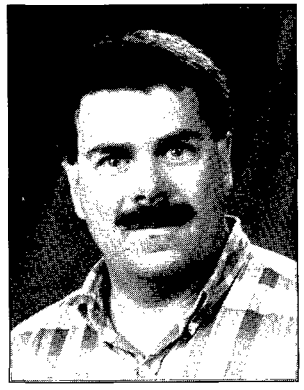
Robert J. Christy, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., The Johns Hopkins University

The primary research interest in my laboratory is the investigation of the genetic and metabolic regulation of gene expression. These studies utilize both *in vitro* and *in vivo* models to analyze gene expression in both adipose and liver.

Using an *in vitro* culture system preadipocyte cells will differentiate into adipocytes in response to a specific hormonal stimuli. This terminal differentiation reflects the process of adipose tissue formation *in vivo* and is mediated through increased rates of transcription of adipose-specific genes. This gene activation leads to a change in the cell morphology and the formation of large lipid droplets in the differentiated adipose cells. Currently, we are examining this process of coordinate gene activation using several lipogenic genes that are activated during the differentiation process. We have found common DNA sequences in these lipogenic gene promoters that are important for gene activation and are now investigating the protein/DNA interactions involved in mediating these adipose-specific responses. We are also interested in understanding the mechanism of adipose cell determination and differentiation during embryogenesis. These studies involve isolating and characterizing novel genes expressed during the early events of adipose cell development and determining their involvement in cell commitment and adipose cell differentiation.

Other studies in the laboratory are investigating the effects of specific diets and hormones on the regulation of lipogenic gene expression in liver and adipose tissue. Under normal nutritional conditions lipogenic genes are expressed at low basal levels; in starved animals the lipogeniic genes are inactivated and lipolytic and gluconeogenic genes are activated. We are studying the changes that occur during this switch from lipogenic gene to gluconeogenic gene expression and how these activities are influenced by specific diearacterization of the Gene and Trans-Activation by the CCAAT/Enhancer Binding Protein. Proc. Natl. Acad. Sci. USA 87:251-255, 1990.



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Christy, R.J., Kaestner, K.H., Geiman, D.E. and Lane, M.D. The CCAAT/Enhancer Binding Protein Gene Promoter: Binding of Nuclear Factors During Differentiation of 3T3-L1 Preadipocytes. Proc. Natl. Acad. Sci. USA 88:2593-2597, 1991.

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Robert A. Clark, M.D.

Professor and Chair, Department of Medicine
M.D., Columbia University

Our laboratory examines the cellular and molecular determinants of activation of the human neutrophil. Two main projects are presently under investigation. The first deals with calcium-mediated signal transduction. Protein components of the system, including the Ca2+ storage protein calreticulin and the inositol trisphosphate-gated Ca2+ release channel, have been cloned and expressed. Current experiments deal with promoter elements and the regulation of expression during myeloid cell differentiation, biosynthesis and post-translational processing, and structural determinants of function using mutagenesis and various expression systems. The second project concerns the respiratory burst oxidase, a multi-component enzyme complex responsible for stimulus-dependent formation of superoxide anion. The several protein constituents have been cloned and we are using both native and recombinant proteins to determine the mechanism for activation. Specific issues include stimulus-dependent phosphorylation, translocation of cytosolic components to membranes, interactions with cytoskeletal elements, the role of domains homologous to src (SH3) and the function of the ras GTP-binding proteins. The overall goal of the studies is to understand, at a molecular level, the neutrophil responses that result in microbial killing and tissue injury.

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Nauseef, W.M., McCormick, S., Renee, J., Leidal, K.G. and Clark, R.A. Functional domain in an arginine-rich carboxy terminal region of p47 phox. J. Biol. Chem. 268:23646-23651, 1993.

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Stendahl, O., Kraus,e K.-H., Kricher, J., Jerstrom, P., Clark, R.A., Carpentier, J.-L. and Lew, D.P. Redistribution of intracellular Ca2+ stores during phagocytosis in human neutrophils. Science 265:1439-1441, 1994.

Jacqueline J. Coalson, Ph.D.

Professor, Department of Pathology
Ph. D., The University of Oklahoma

Work in my laboratory focuses on the role of acute injury on subsequent lung growth and development in the premature baboon. In this unique animal model premature baboons delivered by Caesarean section develop hyaline membrane disease, and if ventilated and treated with oxygen over a prolonged period of time, develop bronchopulmonary dysplasia (BPD). BPD is a serious chronic disease in human infants. Of the four million births annually in the United States, nearly 80,000 will be born significantly premature, and of these, 10,000 will develop BPD. Instillation of surfactant preparations into the lungs of these surfactant-deficient pre-term newborns has dramatically improved acute survival. There is an increased risk for subsequently acquiring BPD, especially in those less than 1,000 grams at birth in which 70 to 80% will develop BPD despite surfactant therapy. These infants exhibit incomplete lung development that requires high levels of barotrauma and supplemental oxygen during mechanical ventilation, leading to protracted injury and abnormal growth of damaged lungs. It is hypothesized that the injury sequence interferes with elastogenesis and secondary crest formation needed for alveolar formation in the immature lung.

Recently, a BPD resource center based at Southwest Foundation for Biomedical Research has been directed by Dr. Jacqueline Coalson, P.I. and by the National Heart, Lung, and Blood Institute to foster innovative research into the mechanisms underlying the development of bronchopulmonary dysplasia. Its goal is to develop a multi-institutional, multidisciplinary effort to address the mechanisms of postnatal lung pathobiology that lead to this chronic lung disease. As part of this study we will determine the effects of several growth modulators on lung growth and development in treated long-term survivors with bronchopulmonary dysplasia. The effect of early preventive therapies on long-term alveolarization, growth, and development of premature animals with and without bronchopulmonary dysplasia will be assessed using specific lung growth parameters at various times following treatment. Research tools being utilized in this study include immunocytochemistry, *in-situ* hybridization, morphometry, and transmission electron microscopy.

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Suzanne A. W. Fuqua, Ph.D.

Associate Professor, Department of Medicine
Ph.D., The University of Texas Health Science Center
at San Antonio

Heat Shock Protein Mechanisms in Breast Cancer Behavior

Although much is known about the transcriptional factors involved in regulating the major heat shock proteins, such as hsp70, very little is known about the factors or promoter elements important in hsp27 induction. We have analyzed the transcriptional regulation of hsp27 expression in human breast cancers with our goal being to discover potential targets for therapeutic intervention by modifying hsp27 levels. Sequencing of a 1.1 kb region of the hsp27 promoter revealed two TATA, two SP1, two AP2, two CAT boxes, two half ERE, and one heat shock element. Furthermore, there are several putative hormone response elements present in this region. We employed DNase I footprint analysis to demonstrate TAT, AP2, and SP1 binding. The estrogen receptor failed to bind to two half ERE sites; however, a novel ERE-binding protein was identified. We have also identified a regulatory element whose negative effect is relieved by further upstream elements. These experiments are important since future therapeutic strategies can be developed which are aimed at these regulatory sites. This type of approach is novel and potentially clinically important ground work for interfering with breast cancer proliferation driven by hsp27.

Estrogen receptor variants in clinical breast cancer.

Several estrogen receptor variants with different functional capabilities have been isolated from clinical specimens. Variants of the estrogen receptor may be associated with poor prognosis and will be the target of a large study to determine its prognostic significance in breast cancer patients. Recent data also suggest that estrogen receptor variants with increased sensitivity to hormone, and increased activity may play a role in early breast carcinogenesis and we are actively pursing this area of research.

Selected Publications

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Oesterreich, S., Weng, C.-N., Qiu, M., Hilsenbeck, S. and Fuqua, S.A.W. Heat shock protein 27 is correlated with growth and drug resistance in human breast cancer cells. Cancer Research, 53:4443-4448, 1993.

Fuqua, S.A.W., Oesterreich, S., Hilsenbeck, S.G., VonHoff, D.D., Eckardt, J. and Osborne, C.K. Heat shock proteins and drug resistance. Breast Cancer Research and Treatment, 32:67-71, 1994.

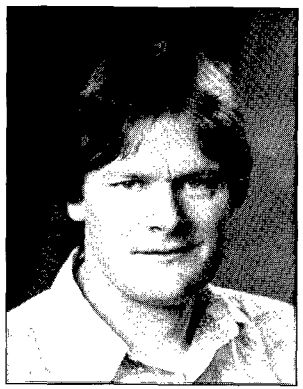
Allred, D.C., O'Connell, P.O. and Fuqua, S.A. Biomarkers in early breast neoplasia. J. Cell Biochem., 17G:125-131, 1993.

Paul D. Gardner, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., University of Pittsburgh

Neurons communicate with each other and with target tissues by way of highly specialized structures known as synapses. I would like to understand the molecular events underlying the formation, maintenance and modification of synapses. Toward this end, the research in my laboratory focuses upon the genetic and epigenetic mechanisms regulating the expression of the genes encoding a family of synaptically localized proteins: nicotinic acetylcholine receptors.

Acetylcholine receptors are the ion channels critical for signal transmission at nerve/muscle synapses as well as at specific synapses within the central nervous system. It is clear that communication between pre- and post-synaptic cells significantly alters the expression of acetylcholine receptor genes and does so, at least in part, at the level of transcription. Our current research is aimed at identifying and characterizing the molecules involved in this phenomenom. We are using these molecular "tools" to gain insight into the signal transduction pathways leading to altered receptor gene expression. These studies will provide important information regarding the general principles of genetic regulation of neurotransmitter receptor expression, as well as advance our understanding of the processes governing neuronal differentiation and development of the nervous system.



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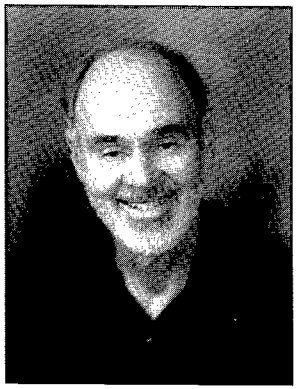
Richard J. King, Ph.D.

Professor, Department of Physiology
Ph.D., University of California, Berkeley

The focus of our laboratory is on the structure and function of pulmonary surfactant, a lipoprotein made by alveolar epithelial cells. This material is released into alveolar fluid and is necessary for preventing alveolar collapse by reducing interfacial tension. Pulmonary surfactant forms a large macromolecular structure which allows its optimal function, and it is clear that this structure is determined by the interaction between unique surfactant proteins and its lipids. Our work has shown that one of the surfactant proteins will only bind to phospholipid domains in a gel (Pß)-phase, and interacts maximally with lamellar arrays containing contiguous domains of gel- and liquid-crystalline (Lα) lipid. Additional work is continuing with investigators using NMR to study changes induced in phospholipid lamellae by another surfactant protein which is highly hydrophobic.

In the last five years it has become evident that the properties and function of pulmonary surfactant are compromised in widespread chronic lung injuries of the newborn and adult which are associated with extended periods of managed ventilation and hyperplastic repair. Because of the evidence that surfactant proteins are important in maintaining the structure of surfactant, we investigated changes in the composition of the surfactant proteins in oxidative stress and found that these proteins are reduced in amounts, of which at least part of the reduction is due to a transcriptional downregulation, and that these effects result in perturbations in the physical properties of surfactant. Our current work is primarily focused on describing the factors responsible for these changes, and we have found that two growth factors not normally produced by adult lung fibroblasts, transforming growth factor-α and hepatocyte growth factor, are synthesized by these cells during oxidative stress. Current work is emphasizing the regulation of these growth factors and their effects on alveolar epithelial cells.



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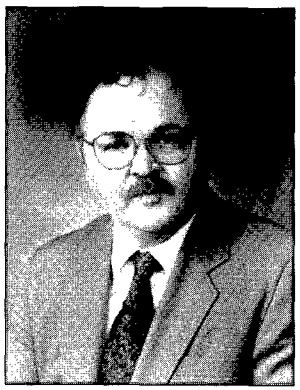
Robert J. Klebe, Ph.D.

Professor, Department of Cellular and Structural Biology
Ph.D., Yale University

Our laboratory is interested in the control of morphogenesis by homeobox genes. The homeobox genes provide the highest level of control during embryonic development. While the molecular genetics of the homeobox genes is now well established, the genes controlled by the homeobox genes are currently unknown. We are currently testing a new theory, proposed by Gerald Edelman, that postulates that the homeobox genes primarily regulate the expression of cell adhesion proteins and their receptors.

We are also interested in a new area, termed "tissue engineering." Tissue engineering involves creating tissues and organs from single cells. We have developed a computerized technique that permits one to microposition cells within a cell diameter on any substratum.



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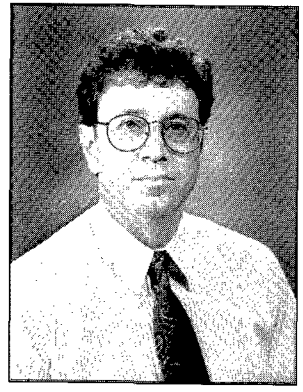
Jeffrey D. Kreisberg, Ph.D.

Professor, Department of Pathology
Ph.D., The University of Maryland School of Medicine

One major research emphasis of my laboratory has been to elucidate the mechanisms of increased extracellular matrix synthesis by mesangial cell cultures exposed to high glucose. Expansion of the renal glomerular mesangium is the structural lesion most closely correlated with developing clinical nephropathy. High glucose stimulates the *de novo* synthesis of diacylglycerol and activates protein kinase C in mesangial cell cultures. Treatment of mesangial cells with agents that activate protein kinase C (e.g., PMA or oleoyl acetyle glycerol) also stimulates extracellular matrix protein synthesis and mRNA expression, indicating an important role for protein kinase C activation in extracellular matrix protein synthesis in cells exposed to high glucose. Experiments are ongoing to determine the enhancer elements in the fibronectin promoter responsive to high glucose. Another major interest of mine has been to understand the factors involved in regulating mesangial cell shape *in vitro.* Mesangial cells exposed to cAMP-elevating agents change shape from flat cells with numerous bundles of stress fibers to highly arborized cells with long thin processes and fragmented stress fibers. These cells are also less tightly adhered to the substratum. Contractile agents for mesangial cells such as vasopressin and thrombin inhibit cAMP induced shape change and adhesion loss. We have determined a significant role for u-PA activation in shape change and are currently studying the role of the vitronectin receptor in shape change.



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Ayo, S.H., Radnik, R.A., Garoni, J.A., Glass, W.F., II and Kreisberg, J.I. High glucose causes an increase in extracellular matrix proteins in cultured mesangial cells. Am. J. Pathol. 136:1339-1348, 1990.

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Eileen M. Lafer, Ph.D.

Associate Professor, Institute of Biotechnology
Ph.D., Tufts University School of Medicine

Synaptic transmission is the basic process which underlies cell-cell communication in the nervous system. The regulation of neurotransmission is an important component of learning and memory. Consequently, there has been considerable interest in understanding how synaptic transmission is effected and regulated at the molecular level. An approach taken by a number of investigators has been to carry out a molecular dissection of the synapse. As part of this effort, we reported the identification, characterization, and cloning of the synapse-specific phosphoprotein F1-20. We found that expression of the F1-20 mRNA and protein are developmentally regulated in a pattern coincident with synaptic maturation, and identified several alternatively spliced isoforms of the F1-20 mRNA. We reported the over-expression of F1-20, and demonstrated its identity with the clathrin assembly protein AP-3. These studies implied a role for AP-3 in synaptic vesicle biogenesis and recycling. We demonstrated that bacterially expressed AP-3 assembles clathrin into cages of a strikingly narrow size distribution. This may be important for the regulation of quantal size during neurotransmission. We also discovered that AP-3 binds to specific inositol polyphosphates with high affinity, and that this binding inhibits clathrin assembly. Inositol polyphosphates have been implicated in the regulation of neurotransmission. It is an intriguing possibility that these effects are mediated through AP-3. We studied the domain organization of AP-3, and found that the N-terminal domain carries out inositol polyphosphate binding while the C-terminal domain carries out clathrin assembly. We are currently determining the specific roles of AP-3 and inositol polyphosphates in synaptic vesicle biogenesis and recycling. A number of other proteins have also been suggested to function in synaptic vesicle biogenesis and recycling, including AP-2, dynamin, amphiphysin, synaptotagmin, p145, and auxilin. We are studying these proteins, with the goal of assigning a role for each protein to a specific step of the synaptic vesicle cycle. We are also examining how this cycle is regulated by protein phosphorylation and inositol polyphosphates. It is expected that these studies will contribute to our understanding of basic mechanisms underlying neurotransmission. This information will be important for elucidating the molecular basis of learning, memory, and neurological disease.

Selected Publications

Zhou, S., Sousa, R., Hrinya Tannery, N. and Lafer, E.M. Characterization of a novel synapse-specific protein: II. cDNA cloning and sequence analysis of the F1-20 protein. J. Neurosci. 12:2144-2155, 1992.

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Eva Lee, Ph.D.

Associate Professor, Institute of Biotechnology
Ph.D., University of California, Berkeley

My long-term research goal is to understand how tumor suppressor genes are involved in the inception, development, and progression of neoplasia. A combination of genetic, biochemical and molecular approaches have been employed to investigate the retinoblastoma tumor suppressor (Rb) gene and the p53 gene. Our current studies are centered on three major areas: (1) the developmental role and tissue-specific functions of the Rb gene; (2) cooperation among Rb, p53, Bcl-2, CKI, etc. in cell cycle control, apoptosis responses and tumor predisposition; (3) a refinement of animal models by tissue-specific inactivation of the tumor suppressor genes in order to address the effects of hormones, growth factors, and environmental factors on tumorigenesis in predisposed mice.

Rb is ubiquitously expressed and numerous studies indicate that Rb is an important negative regulator of cell cycle G1 to S progression. Yet, human carriers are mainly predisposed to retinoblastoma. We have generated mouse strains carrying a mutated Rb gene using gene targeting techniques. Rb-deficient mouse embryos suffer abnormalities in the nervous system, eyes, and erythrocytes and die before embryonic day 16.0. Rb-deficient neuronal cells fail to exit the cell cycle or to terminally differentiate. Using the Rb-deficient animal model, we are investigating genes whose altered expression mediate Rb activities seen in the nervous system. Whether expression of Rb target genes is subjected to regulation by specific Rb protein complexes are being investigated. Our goals for these studies are to decipher the tissue-specific function of the Rb gene and to provide insights into specific tumor predisposition by Rb inactivation. To provide viable mice models for addressing Rb functions in specific tissue, we are using the bacteriophage Cre recombinase to carry out tissue-specific inactivation of Rb and p53 genes in mice. Our initial studies will focus on specific inactivation of these genes either in neurons or in mammary epithelial cells. After our goal of defining specific tumor predisposition in mice is achieved, we will then study how tumor formation is affected by environmental and physiological factors. These studies will illustrate how genetic and epigenetic factors contribute to tumorigenesis.

Selected Publications

Lee, E. Y.-H. P., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.-C., Herrup, K., Lee, W.-H. and Bradley, A. Mice deficient for RB are nonviable and show defects in neurogenesis and hematopoiesis. Nature 359:288-294, 1992.

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Wen-Hwa Lee, Ph.D.

Professor and Director, Institute of Biotechnology
Ph.D., University of California, Berkeley

The major research interest of my laboratory is to unravel the molecular basis of cancer formation. During the past ten years, we have isolated the retinoblastoma (Rb) gene and explored the molecular basis of cancer suppression by this gene. Given the theoretical cancer suppressing activity of the Rb gene, an assay system for Rb gene function was developed by introducing the gene into cultured tumor cells with inactivated endogenous Rb genes. We have consistently found that replacement of Rb protein in these tumor cells suppresses their tumorigenicity. These results — replacement of suppressor genes in tumor cells — provide a critical foundation to consider a novel strategy for the treatment of clinical malignancy.

To further establish this protocol, we have developed a mouse model in which one allele of Rb is inactivated by the method of homologous recombination in embryonic stem cells. Our results indicate that mice without normal Rb protein expression are embryonic non-viable due, probably, to ectopic mitosis leading to cell death in neuronal or hematopoetic systems. With only one copy of the Rb gene, mice produce pituitary intermediate lobe tumors in nearly 100% at the age of about 10 months. A gene therapy approach using Rb was developed and has been shown to be effective in prolonging the life span of tumor-bearing mice. The success of developing this mouse model will provide important insight toward the clinical treatment of cancer in humans.

To understand the basic mechanism of Rb function, we have identified the Rb gene product as a nuclear phosphoprotein, pp110^{RB}. This protein has two fundamental biochemical functions: one is to sequester transcriptional factors such as E2F which are crucial for cell cycle progression; the other is to activate another group of transcriptional factors such as C/EBP which are important for differentiation. Based on these two distinct activities, Rb is a key regulator of cellular responses to external signals for cell growth and differentiation. Similarly, we are exploring the molecular basis of the breast carcinoma gene (BRCA1) following the Rb paradigm.

Selected Publications

Lee, E.Y.-H.P., Chang, C.-Y., Hu, N., Wang, Y.-C. J., Lai, C.C., Herrup, K., Lee, W.-H. and Bradley, A. Mice deficient for RB are nonviable and show defects in neurogenesis and hematopoiesis. Nature 359:288-294, 1992.

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Chen, Y., Chen, C.-F., Riley, D.J., Allred, D.C., Chen, P.-L., Von Hoff, D., Osborne, C.K. and Lee, W.-H. Aberrant subcellular localization of BRCA1 in breast cancer. Science, 270:789-791, 1995.

Chen, P.-L., Riley, D.J., Chen-Kiang, S. and Lee, W.-H. Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc. Natl. Acad. Sci., 93:465-470, 1995.

Bettie Sue Siler Masters, Ph.D.

Professor, Department of Biochemistry
Ph.D., Duke University

My research interests fall into three general categories: 1) Functionally characterizing the physical properties and role of NADPH-cytochrome c (P-450) reductase in microsomal electron transport using spectrophotometric titrations, fluorescence polarization and time-resolved energy transfer measurements, electron paramagnetic resonance (ESR), nuclear magnetic resonance (NMR), and crystallographic techniques on the preparation capable of reducing cytochrome P-450. Wild type and mutant preparations of the rat liver reductase expressed in *E. coli* are being studied with the purpose of determining structural properties of site-directed mutants. 2) Determining the structure-function relationships of cerebellar nitric oxide synthase by a variety of biophysical techniques and microdissection of the protein is being performed using molecular cloning techniques. 3) Determining the cellular origin, biochemical mechanisms of formation, and role of hydroxylated prostaglandins E and F and ω-hydroxyeicosatetraenoic acids (HETEs formed by cytochromes P450) in liver, kidney, and lung. Structure-function relationships are being studied by chimeric constructions and site-directed mutagenesis of homologuous cDNAs.

Selected Publications

Sheta, E., McMillan, K. and Masters, B.S.S. Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase. J. Biol. Chem. 269:15147-15153, 1994.

Narayanasami, R., Horowitzm, P.M. and Masters, B.S.S. Flavin-binding and protein structural integrity studies in NADPH-cytochrome P450 reductase are consistent with the presence of distinct domains. Arch. Biochem. Biophys. 316:267-274, 1995.

McMillan, K. and Masters, B.S.S. Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides. Biochemistry 34:3686-3693, 1995.

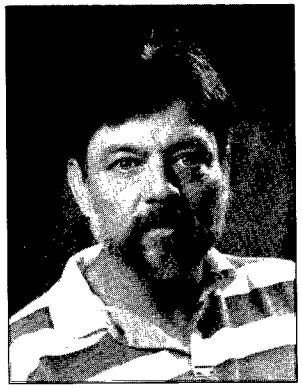
Djordjevic, S., Roberts, D.L., Wang, M., Shea, T., Camitta, M.G.W., Masters B.S.S. and Kim, J.J.P. Crystallization and preliminary x-ray studies of NADPH-cytochrome P450 reductase. Proc. Natl. Acad. Sci., USA 92:3214-3218, 1995.

Roman, L.J., Sheta, E.A., Martasek, P., Gross, S.S., Liu, Q. and Masters, B.S.S. High level expression of functional rate neuronal nitric oxide synthase in *Escherichia coli.* Proc. Natl. Acad. Sci., USA 92:8482-8432, 1995.

Salerno, J.C., Frey, C., McMillan, K., Williams, R., Masters, B.S.S. and Griffith, O.W. Nitric oxide synthase: Characterization by electron paramagnetic resonance of the interactions of L-arginine and L-thiocitrulline with the heme cofactor region. J. Biol. Chem., in press, 1995.

Eduardo A. Montalvo, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., The University of Texas Health Science Center at San Antonio



My laboratory is interested in dileneating the molecular mechanisms of virus latency. Latency is a reversible nonproductive infection which has been developed by some viruses as a strategy to prevent elimination by the host immune system. Thus, a long-term chronic infection is established. The system which we use to study virus latency is the Epstein-Barr virus (EBV) system. EBV, a member of the herpesvirus family, is the etiological agent of infectious mononucleosis and is associated with various malignancies including Burkitt's lymphoma and nasopharyngeal carcinoma. The virus replicates in oropharyngeal epithelial cells and establishes latency in resting B lymphocytes. *In vitro*, latency in infected B cells can be disrupted by several chemical and biological agents. Disruption of latency is controlled by the EBV BZLF1 gene which encodes an immediate-early protein that functions as a transcriptional activator. Because the BZLF1 gene represents the latent/lytic switch in the virus life cycle, we have focused on (1) identifying regulatory elements in the BZLF1 promoter which either repress or activate transcription, (2) defining the factors which regulate at these sites and (3) defining post-translational regulation of these factors. Several important *cis*-acting elements in the EBV BZLF1 promoter have now been identified and include four phorbol ester (TPA) responsive elements, two anti-immunoglobulin responsive elements and six *cis*-acting negative elements. We have identified at least two distinct proteins which regulate BZLF1 transcription, YY1 and an ATF family member. YY1 is a ubiquitous protein which binds to *cis*-acting negative elements in the BZLF1 promoter and negatively regulates transcription. Binding of YY1 to the promoter is abolished by TPA, an inducer of EBV lytic replication. The ATF protein binds to a key TPA and anti-immunoglobulin responsive element in the promoter and positively regulates BZLF1 transcription. A third protein, Sμbp-2, was cloned by probing an expression library with sequences corresponding to two TPA responsive elements. Sμbp-2, a member of a new superfamily of putative helicases, is a 130 kDa protein which has also been shown to possess transactivating transcriptional activity.

The future goal of the laboratory is to determine how these proteins affect EBV gene expression and to determine the mechanisms which regulate the transcriptional activity of these regulatory proteins.

Selected Publications

Montalvo, E.A. and Grose, C. Neutralization epitope of varicella-zoster virus on native glycoprotein gp118 (VZV glycoprotein gpIII is selectively phosphorylated by a virus-induced protein kinase. Proc. Natl. Acad. Sci, USA 83:8967-8971, 1986.

Montalvo, E.A., and Grose, C. Assembly and processing of the disulfide-linked varicella-zoster virus glycoprotein gpII (140). Journal of Virology 61:2877-2884, 1987.

Montalvo, E.A., Shi, Y., Shenk, T.E. and Levine, A.J. Negative regulation of the BZLF1 promoter. Journal of Virology 65:3647-3655, 1991.

Montalvo, E.A., Cottam, M., Hill, S. and Wang, Y-C. J. YY1 binds to and regulates *cis*-acting negative elements in the Epstein-Barr virus BZLF1 promoter. Journal of Virology 69:4158-4165, 1995.

Mary Pat Moyer, Ph.D.

Professor, Department of Surgery
Ph.D., The University of Texas at Austin

My research group focuses on "translational" studies that are intended to mesh basic research and clinical applications. In close collaboration with clinicians, the group has done pioneering work to develop *in vitro* models of many types of human cells to study pathophysiology at the cellular and molecular levels. We have developed valuable cell lines, including those from organs whose cells are difficult to culture, such as the gastrointestinal (GI) tract. From fresh tissue specimens, we have banked cells and documented reference tissues from multiple organ sites. We have improved technologies to generate human gene banks useful to study the genetic basis of diseases, such as cancer and diabetes. Basic and clinical research studies in oncology (GI, oral and other organ sites) are being pursued with these cell models. The role of defined genes, growth factors, and hormones in various cancer-derived, premalignant and normal cells is being explored in conjunction with work to identify markers and regulatory mechanisms of cell growth, malignant transformation and differentiation. Other work is focused on tissue modeling and cell-microbe interactions in infectious disease. Unique human cell models have been developed to understand latency and persistence of the human immunodeficiency virus, HIV. In the context of that work, the first *in vitro* model to study Kaposi's sarcoma has been developed by our group, as have models of HIV persistence, microbial co-factors, and cell regulation in GI and other non-lymphoid human cells.

Selected Publications

Moyer M.P., Ramirez A., Woolf A., Huot R.I. and McKee-Saurez M.S. HIV infection of human gastrointestinal submucosal cells: An *in vitro* model of Kaposi's sarcoma. In Vitro Cell. Devel. Biol. 27A:337-344, 1991.

Goodwin T.J., Schroeder W.F., Wolf D.A. and Moyer M.P. Rotating-wall vessel coculture of small intestine as a prelude to tissue modeling: Aspects of simulated microgravity. Proc. Soc. Exp. Biol. Med. 202:181-192, 1993.

Wall F.E., Henkel R.D., Stern M.P., Jenson H.B. and Moyer M.P. An efficient method for routine Epstein-Barr virus immortalization of human B Lymphocytes. In Vitro Cell Devel. Biol.: Animal, in press, 1995.

Stauffer J.S., Manzano L.A., Balch G.C., Merriman R.L., Tanzer L.R. and Moyer M.P. Development and characterization of normal colonic epithelial cell lines derived from normal mucosa of patients with colon cancer. Am. J. Surg. 169:190-195, 1995 .

Moyer M.P. Tumor Cell Culture. P.K. Vogt and I. Verma (eds). Meth. Enzymology, in press, 1995.

Gregory R. Mundy, M.D.

Professor, Department of Medicine
M.D., The University of Tasmania

Our program involves three main areas of investigation. We investigate the biology of the osteoclast using both *in vitro* and *in vivo* techniques. These studies involve examination of the life cycle of the osteoclast, including identification of the cell of origin, gene expression during osteoclast differentiation, the growth regulatory factors and cytokines which are responsible for osteoclast formation and regulation of osteoclast apoptosis. The role of growth regulatory factors and cytokines in common bone diseases such as osteoporosis, Paget's disease and cancers metastatic to the skeleton is also an active area of investigation.

Our second major area of investigation is the regulation of bone formation and gene expression during osteoblast differentiation. In particular, we are interested in the role of growth regulatory factors in promoting bone cell differentiation and bone mineralization. Our investigators are examining way in which bone differentiation can be modulated for the purpose of developing drugs for the treatment of the common diseases of bone loss.

Our third main area of investigation involves the effects of tumors on the skeleton. Tumors affect the skeleton in two ways, either by causing osteoblastic or bone forming metastases or more commonly destructive or osteolytic metastases. We examine the molecular mechanisms involved in the multi-step process of tumor cell metastasis to bone, and have devised pharmacologic approaches to preventing or reversing this process. We are interested in the role of bone as a fertile environment for the growth of tumor cells, and the molecular mechanisms which are responsible.

Selected Publications

Yoneda T., Alsina M.M., Chavez J.B., Bonewald L., Nishimura R. and Mundy G.R. Evidence that tumor necrosis factor plays a pathogenetic role in the paraneoplastic syndromes of cachexia, hypercalcemia, and leukocytosis in a human tumor in nude mice. J. Clin.Invest. 67:977-985, 1991.

Boyce B.F., Yoneda T., Lowe C., Soriano P. and Mundy G.R. Requirement of pp60^{0} expression of osteoclasts to form ruffled borders and resorb bone. J. Clin. Invest. 90:1622-1627, 1992.

Mbalaviele, G., Chen, H., Boyce, B.F., Mundy, G.R. and Yoneda, T. The role of cadherin in the generation of multinucleated osteoclasts from mononuclear precursors in murine marrow. J. Clin. Invest. 95:2757-2765, 1995.

Sasaki, A., Boyce, B.F., Story, B., Wright, K.R., Chapman, M., Boyce, R., Mundy, G.R. and Yoneda, T. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res. 55:3551-3557, 1995.

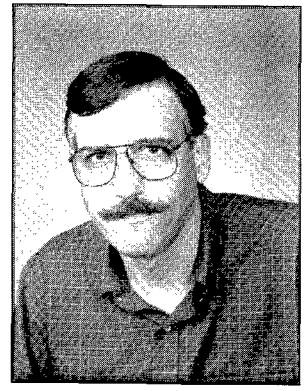
Dallas, S.L., Miyazono, K., Skerry, T.M., Mundy, G.R. and Bonewald, L.F. Dual role for the latent transforming growth factor beta binding protein (LTBP) in storage of latent TGFβ in the extracellular matrix and as a structural matrix protein. J. Cell Biol., in press.

Peter O'Connell, Ph.D.

Associate Professor, Department of Pathology
Ph.D., Brandeis University

My research concerns the identification of genes that cause human diseases and neoplasia. We also carry out genetic and physical mapping of human chromosomes as part of the Human Genome Project. In some instances, we study families segregating Mendelian disorders in order to identify the location of the disease-causing gene. Once a map position is known, genes expressed within the target regions can be isolated and characterized for mutations. At present, we are studying families with inherited predispositions to neurological disorders, breast cancer, and non-insulin dependent diabetes mellitus (NIDDM, or type 2 diabetes).

The technology used in these gene mapping studies also permits analysis of formalin-fixed, paraffin-embedded biopsy specimens from human neoplasia. My laboratory is studying *de novo* genetic changes present in these neoplasia in order to help identify genetic loci important in cancers of the breast, prostate, and stomach. These studies will improve classification and diagnosis of these disorders, and aid in the isolation of genes involved in tumor progression.



Selected Publications

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Lemons, R.S., Eilender, D., Waldman, R., Rebentisch, M., Frej, A-K., Ledbetter, D.H,. Willman, C., McConnell, T. and O'Connell, P. Cloning and characterization of the t(15;17) translocation breakpoint region in acute promyelocytic luekemia. Genes Chromosom. Cancer 2:79-87, 1990.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K. Culver, M., Carey, C.J., Copeland, N.G., Jenkins, N.A., White, R. and O'Connell, P. Deletions and translocations interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62:187-192, 1990.

Shannon, K.M., O'Connell, P., Martin, G.A., Paderanga, D., Olson, K., Dinndorf, P. and McCormick, F. Loss of the NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeliod disorders. New Eng. J. Med. 330:597-601, 1994.

Ryan, S.G., Buckwalter, M.S., Lynch, J.W., Handford, C.A., Segura, L., Shaing, R., Wasmuth, J.J., Camper, S.A., Schofield, P. and O'Connell, P. A missence mutation in the gene edcoding the α1 subunit of the inhibitory glycine receptor in the spasmodic mouse. Nat. Genet. 7:131-135, 1994.

C. Kent Osborne, M.D.

Professor, Department of Medicine
M.D., The University of Missouri

Our laboratory has major programs in two distinct areas. The first involves identification of the mechanisms by which breast cancers develop resistance to tamoxifen—the antiestrogen most used in breast cancer treatment. We have shown that one mechanism is through the acquired ability of the tumor to be stimulated by, rather than inhibited by, tamoxifen. We are identifying and characterizing ancillary proteins that may interact with the estrogen receptor in the genesis of the tamoxifen-stimulated phenotype. An alternative hypothesis is based on our observation that tamoxifen treatment induces cellular oxidant stress that may, through the induction of *fos* and *jun* family members, result in a proliferative response. Our group is attempting to inhibit the development of resistance by antioxidant treatment of mice bearing human breast cancers and through the expression of the dominant negative inhibitor of *jun* in these tumors.

The program involves growth regulation of human breast cancer. We are focusing on several growth factors and receptor families in the pathogenesis of breast cancer. Using a transgenic mouse strain developed in our laboratory, we are investigating the role of TGF-α in the induction of mammary carcinomas. In addition to identifying other genes that are required for development of the malignant phenotype, we are also investigating unique methods of blocking this pathway through the use of TGF-α vaccines. Finally, the importance of the expression of this family of growth factors and receptors is being evaluated in a panel of human breast tissue samples representing various stages in the evolution of disease from normal to hyperplastic and finally to malignancy.

Selected Publications

Osborne, C.K., Coronado-Heinsohn, E.B., Hilsenbeck, S.G., McCue, B.L., Wakeling, A.E., McClelland, R.A., Manning, D.L. and Nicholson, R.L.. Pure steroidal antiestrogens are superior to tamoxifen in a model of human breast cancer. J. Natl. Cancer Inst. 87:746-750,1995.

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Allred, D.C., O'Connell, P., Fuqua, S.A.W. and Osborne, C.K. Immunoourtochemical studies of early breast cancer evolution. Breast Cancer Res. Treat. 32:13-18, 1994.

Osborne, C.K., Jarman, M., McCague, R., Coronado, E.B., Hilsenbeck, S.G. and Wakeling, A.E. The importance of tamoxifen metabolism in tamoxifen-stimulated breast tumor growth. Cancer Chemother. Pharmacol. 34:89-95, 1994.

Stein, D., Wu, J., Fuqua, S.A.W., Roonprapunt, C., Yajnik, V., E'Eustachio, P., Moskow, J.J., Buchberg, A.M., Osborne, C.K. and Margolis B. The SH2 domain protein GRB-7 is coamplified, overexpressed and in a tight complex with HER2 in breast cancer. EMBO J. 13:1331-1340, 1994.

David S. Papermaster, M.D.

Professor, Departments of Pathology and Cellular and Structural Biology
M.D., Harvard University

My laboratory addresses two questions: 1) How do cells distribute their plasma membrane proteins so that one end of the cell is different from the other? 2) What is the molecular pathway(s) regulating apoptosis in inherited retinal degenerations? To investigate the first question, we are testing the hypothesis that discrete post-Golgi membranes transport newly synthesized proteins toward their site of function on the plasma membrane. To study this problem, one needs a favorable cell. The rod photoreceptor in the frog retina has proven to be a most fortunate choice. It synthesizes 80,000 new molecules of rhodopsin per minute and transports it through the Golgi and then to the outer segment where rhodopsin is assembled into light-sensitive disk-shaped membranes. We have purified the post-Golgi membranes of photoreceptors and showed that their movement is not dependent on continuous opsin release from the Golgi since their passage to the outer segment progresses in the presence of drugs which disrupt glycoprotein transport out of the Golgi. Many other minor proteins have now been identified as components of these post-Golgi membranes. Two of these, are αA- and αB-crystallin which were previously thought to be lens-specific proteins. Could they be a new class of cytoskeletal proteins or do they act as heat-shock proteins/molecular chaperonins? Several small GTP binding proteins (Mr=3D21-25 kD), especially rabs 6, 8, and 10, appear to be unique to the membranes of this compartment. We are currently seeking to interrupt their function in order to investigate their role in membrane transport. These studies are part of a long-term investigation of normal and dystrophic photoreceptors. The second question arises from our discovery that photoreceptors die by apoptosis in several forms of inherited retinal degenerations, including retinitis pigmentosa in man. A new model under active study involves transgenic mice bearing various genes under the control of promoters that lead to high levels of expression in terminally differentiating photoreceptors. Some of these, e.g., HPV16 E7, act via their binding to the retinoblastoma protein and may represent the consequences of inappropriate entry of the cells into cell division after they normally become post-mitotic neurons.

Selected Publications

Deretic, D., Morrison, H., Aebersold, R.H. and Papermaster, D.S. alpha A and alpha B-crystallin in the retina: Association with the post-Golgi compartment of frog retinal photoreceptors. J. Biol. Chem. 269:16853-16861, 1994.

Howes, K.A., Ransom, N., Papermaster, D.S., Lasudry, J.G.H., Albert, D.M. and Windle, J.J. Apoptosis or retinoblastoma: Alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes and Devel. 8:1300-1310, 1994.

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Deretic, D. and Papermaster, D.S. The role of small G-proteins in the transport of newly synthesized rhodopsin. In "Progress in Retinal and Eye Research," N. Osborne and G. Chader, eds. Pergamon Press, Oxford, NY, (1995) pp 249-265.

Arlan Richardson, Ph.D.

Professor, Department of Physiology
Ph.D., University of Oklahoma State

The focus of my research is to understand what role changes in gene expression play in aging and senescence. Specific emphasis is placed on studying how aging and dietary restriction alter the transcriptional regulation of specific genes and the ability of cells to repair damage in specific regions of genes. Most of my research involves studying gene expression or DNA repair in isolated hepatocytes or spleen lymphocytes. We are currently studying the transcription factors that regulate the expression of the following genes: hsp70, a heat shock protein that plays a critical role in protecting cells from hyperthermia and other stresses, and catalase, an antioxidant enzyme that protects cells from damage arising from oxygen free radicals. We are also using transgenic mice to identify genes that play a critical role in aging. For example, we are constructing transgenes that can be used to produce transgenic mice that overexpress the antioxidant enzymes catalase and Cu/Zn-superoxide dismutase. These transgenic mice will allow us to determine the role free radicals play in the aging process and the occurrence of age-related diseases. In addition, we have produced transgenic mice that overexpress metallothionein, which is a small, cysteine-rich protein that protects cells from heavy metals, oxygen radicals, radiation, and alkylating agents. These transgenic mice will be used to define the role metallothionein plays in protecting animals from carcinogens and oxygen stress.

Selected Publications

Richardson, A., Butler, J.A., Rutherford, M.S., Semsei, I., Gu, M.Z., Fernandes, G. and Cheung, W.H. Effect of age and dietary restriction on the expression of a 2u-globulin. J. Biol. Chem. 262:12821-12825, 1987.

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Heydari, A., Wu, B., Takahashi, R., Strong, R. and Rictardson, A. Expression of heat shock protein 70 is altered by age and diet at the level of Transcription. Mol. Cell. Biol. 13:2909-2918, 1993.

Takahashi, R., Heydari, A.R., Gutsmann, A., Sabia, M. and Richardson, A. The heat shock transcription factor in liver exists in a form that has DNA binding activity but no transcriptional activity. Biochem. Biophys. Res. Commun. 201:552-558, 1994.

G. David Roodman, M.D., Ph.D.

Professor, Department of Medicine
M.D., University of Kentucky College of Medicine
Ph.D., University of Kentucky

My laboratory has been interested for the last decade in factors controlling the proliferation and differentiation of hematopoietic progenitors. In particular, we have focused our efforts on studying the osteoclast, the primary cell involved in bone resorption. We have identified the early and more committed precursors for these cells and developed marrow culture techniques for obtaining osteoclasts from normal patients and from those with pathologic states associated with increased bone turnover *in vitro*. In addition, we have made cDNA libraries from these osteoclast-like cells formed *in vitro*, as well as from osteoclasts isolated from giant cell tumors of bone. Our goal is to understand the molecular events involved in differentiation and commitment of these early precursors to cells in the osteoclast lineage. We have also used these libraries to identify autocrine factors produced from osteoclasts that enhance bone formation and differentiation. Most recently, we have undertaken studies using differential display PCR-based techniques and differential hybridization techniques to examine the genes responsible for hypercalcemia in patients with multiple myeloma. In addition, we are trying to produce osteoclast cell lines from transgenic mice in which the SV40 large T antigen is targeted to the osteoclast.

Selected Publications

Demulder, A., Takahashi, S., Singer, F.R., Hosking, D.J. and Roodman, G.D. Abnormalities in osteoclast precursors and the marrow accessory cells in Paget's Disease. Endocrinology 133:1978-1982, 1993.

Takahashi, S., Goldring, S., Katz, M., Hilsenbeck, S., Williams, R. and Roodman, G.D. Downregulation of calcitonin receptor mRNA expression by calcitonin during osteoclast-like cell differentiation. J. Clin. Invest. 95:167-171, 1995.

Takahashi, S., Reddy, S.V., Chirgwin, J.M., Devlin, R., Haipek, C., Anderson, J. and Roodman, G.D. Cloning and identification of Annexin II as an autocrine/paracrine factor that increases osteoclast formation and bone resorption. J. Biol. Chem. 269:1-6, 1994.

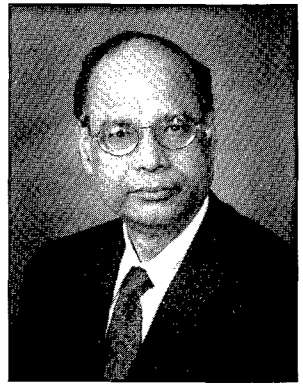
Reddy, S.V., Hundley, J.E., Windle, J.J., Alcantara, O., Leach, R.J., Boldt, D.H. and Roodman, G.D. Characterization of the mouse tartrate-resistant acid phosphatase (TRAP) genepromoter. J Bone Miner Res., in press.

Takahashi, S., Reddy, S.V., Dallas, M., Chou, J.Y. and Roodman, G.D. Development and characterization of a human marrow stromal cell line that enhances osteoclast-like cell formation. Endocrinology 136:1441-1449, 1995.

Arun K. Roy, Ph.D.

Professor, Department of Cellular and Structural Biology
Ph.D., Wayne State University

The androgen receptor is a ligand-activated transcription factor that guides the molecular events associated with male reproductive functions. It is normally expressed in various cell types in a spatio-temporal fashion. The temporal aspect of androgen receptor gene expression is genetically programmed and overlaps with programs that control the processes of development, maturation and aging. An overall understanding of the regulatory events for the spatio-temporal changes in androgen sensitivity and expression of the androgen receptor gene is therefore expected to provide a deeper insight into the mechanism of the multicomponent temporal control associated with aging. Furthermore, results of these studies may lead to better approaches for management of age-dependent disorders of androgen action such as prostatic hypertrophy and hyperplasia. In our laboratory we have been exploring the molecular basis of the age-dependent changes in androgen responsiveness of the liver. Androgen sensitivity in the liver starts to be evident around puberty (~35 to 40 days), reaches a peak level at 75-85 days and gradually declines after ~150 days, with an almost total loss by 750-800 days. These age-related changes in androgen sensitivity are associated with temporal expression of the androgen receptor gene and certain steroid inactivating enzymes [1,2]. In order to understand the molecular events responsible for the age-dependent changes in target cell sensitivity of androgenic hormones, we have cloned and characterized the androgen receptor gene promoter [3]. Both functional mapping of the promoter fragments through transfectional assays and structural mapping through DNA-protein interactions and footprinting analysis have led to the identification of two age-dependent regulatory elements on the androgen receptor gene. One of these elements binds a novel and ubiquitous age-dependent factor (ADF) which serves as a positive regulator [4]. Cloning and characterization of ADF are currently in progress. We have also discovered that aging is associated with a marked upregulation of a member of the NF-kB/Rel family of transcription factors that acts as a repressor for the androgen receptor gene [5]. The NF-kB/Rel family of transcription factors generally functions as positive regulators of gene transcription. The mechanism of this unconventional negative regulatory function of the p50/p50 homodimer of NF-kB, mediated through topological alteration of the DNA helix and protein-protein interactions, is also the subject of our present interest.



Selected Publications

Mancini, M.A,. Song, C.S., Rao, T.R., Chatterjee, B. and Roy, A.K. Spatio-temporal expression of estrogen sulfotransferase within the hepatic lobule of male rats: Implication in androgen action. Endocrinology 131:1541, 1992.

Demyan, W.F., Song, C.S., Kim, D.S., Her, S., Gallwitz, W., Rao, T.R., Slomczynska, M., Chatterjee, B. and Roy, A.K. Estrogen sulfotransferase of the rat liver: cDNA cloning, age- and sex-specific regulation of mRNA. Mol. Endocrinol. 6:589, 1992.

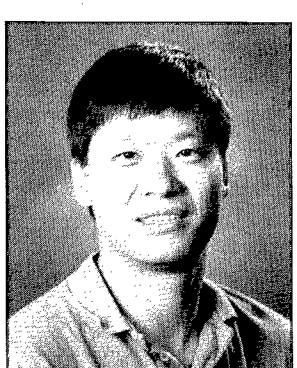
Song, C.S., Her, S., Choi, S.J., Slomczynska, M., Roy, A.K. and Chatterjee, B. A distal activation domain is critical in the regulation of the rat androgen receptor gene promoter. Biochem. J. 294:779, 1993.

Supakar, P.C., Song, C.S., Jung, M.H., Slomczynska, M.A., Kim, J.-M., Vellanoweth, R.L., Chatterjee, B. and Roy, A.K. A novel regulatory element associated with age-dependent expression of the rat androgen receptor gene. J. Biol. Chem. 268:26400, 1993.

Supakar, P.C., Jung, M.H., Song, C.S., Chatterjee, B. and Roy, A.K. Nuclear factor kB functions as a negative regulator for the rat androgen receptor gene and NF-kB activity increases during age-dependent desensitization of the liver. J. Biol. Chem. 270:837, 1995.

Edward Seto, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., University of California, San Francisco



The primary focus of research in our laboratory is on eukaryotic transcription regulation, with an emphasis on cellular and viral transcription factors.

1. *Transcription Factor YY1.* Regulation of eukaryotic mRNA transcription is governed by DNA sequence elements that serve as binding sites for sequence-specific transcription factors. We have previously shown that a binding element for the transcription factor YY1 can activate or repress transcription. Given the diverse effects of the YY1 protein, it seems likely that its function depends on interaction with other cellular transcription factors. The overall hypothesis to be evaluated in this project is that the transcription factor YY1 is an important gene regulator, and its ability to activate and repress transcription is a result of its ability to selectively interact with different cellular proteins. The goal is to understand the mechanism by which YY1 affects transcription, with particular emphasis on studying YY1-binding proteins.

2. *The Hepatitis B Virus Trans-Activator Protein X.* The hepatitis B virus (HBV) is a small, human DNA virus that causes acute and chronic hepatitis and is strongly associated with the development of primary hepatocellular carcinoma. In addition to the genes that code for the major structural proteins, and a gene that codes for the viral polymerase, the HBV genome contains an open reading frame that codes for a protein of 154 amino acids, termed X. Although it has been demonstrated that the X-gene product is a transcriptional activator, little is known about its mechanism of action, its role in the viral life cycle, and its contribution to hepatocellular carcinoma. The focus of this project is to understand, at the molecular level, the mechanism by which the X protein activates gene expression.

Selected Publications

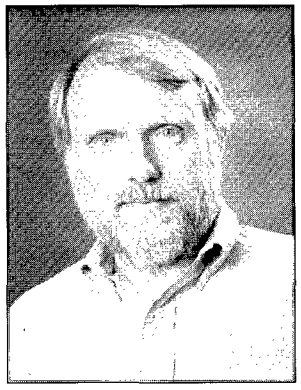
Seto, E., Mitchell, P.J. and Yen, T.S.B. *Trans*-Activation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature 344:72-74, 1990.

Seto, E., Shi, Y. and Shenk, T. YY1 is an initiator sequence-binding protein that directs and activates transcription *in vitro.* Nature 354:241-245, 1991.

Seto, E., Lewis, B. and Shenk, T. Interaction between transcription factors Sp1 and YY1. Nature 365:462-464, 1993.

L. Dave Sharp, Ph.D.

Associate Professor, Institute of Biotechnology
Ph.D., University of Arkansas for Medical Science



Homeobox proteins are important gene regulators that control developmental programs in multicellular organisms from sponges to human beings. The developmentally regulated POU-homeobox protein, Pit-1, is a transactivator of the growth hormone, prolactin and TSHβ genes in mammals. Mutations of the gene encoding Pit-1 results in dwarf mice whose anterior pituitaries lack three of the five cell-types, lactotropes (PRL producers), somatotropes (GH producers) and thyrotropes (thyroid stimulation hormone producers). A goal is to determine the molecular genetic mechanism that restricts GH and PRL expression during development. A hypothesis under test is that Pit-1 transcription activation is dependent on flexible contacts with the basal transcription apparatus in the context of the specific and well conserved prolactin and growth hormone promoter geometry. The hypothesis is also being tested by site-directed mutagenesis of the activation domain of Pit-1 and *in vivo* and *in vitro* affinity screening of cDNA libraries to identify Pit-1-associated proteins.

The other major project in the lab involves understanding the nuclear function of Pit-1. Immunofluoresence and biochemical fractionation showed that Pit-1 is partitioned in the nucleus both spatially and in terms of solubility. Our data are consistent with a model that describes a dynamic equilibrium established for Pit-1 that involves DNA- and protein-binding. Mapping results indicate that the POU-specific domain in the DNA-binding region of Pit-1 is responsible for the observed nuclear partitioning. Projects are underway to assay the functional significance of nuclear partitioning. The work is also a test of a larger principal concerning non-membrane-mediated compartmentation of biological systems as a mechanism for insuring physiologically effective function, in this case transcription.

Selected Publications

Cao, Z., Barron, E.A. and Sharp, Z.D. Prolactin upstream factor I (PUF-I) mediates cell-specific transcription. Mol. Cell. Biol. 8:5432-5438, 1988.

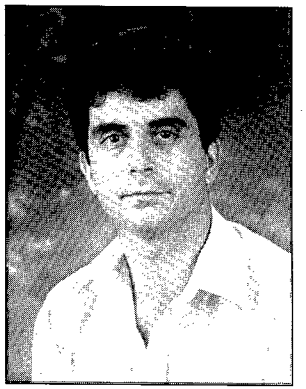
Smith, K.P. and Sharp, Z.D. A Pit-1 binding site 3' to the transcription start site inhibits transcription elongation *in vitro*. Biochem. Biophysic. Res. Comm. 177:790-796, 1991.

Smith, K.P., Liu, B., Scott, C. and Sharp, Z.D. Transcription factor Pit-1 exhibits a unique promoter spacing requirement for activation and synergism. J. Biol. Chem. 270: 4484-4491, 1995.

Sharp, Z.D. Rat Pit-1 stimulates transcription *in vitro* by influencing pre-initiation complex assembly. Biochem. Biophys. Res. Comm. 206:40-45, 1995.

Rui J. Sousa, Ph.D.

Assistant Professor, Department of Biochemistry
Ph.D., The University of Pittsburgh



Dr. Sousa's laboratory studies the structures and mechanisms of nucleic acid polymerases using two distinct but complementary approaches. X-ray crystallography is used to obtain information about the three-dimensional structures of the polymerases and *in vitro* characterization of mutant enzymes is used to correlate structure with function and to elucidate mechanism. The development of mechanistic hypotheses is greatly helped by recent discoveries which have revealed that polymerases of distinct classes with little sequence similarity have very similar 3-dimensional structures. The polymerase studied most extensively in Dr. Sousa's laboratory is the DNA directed RNA polymerase from bacteriophage T7. This relatively small (100 kD) monomeric polymerase can execute all of the steps in promoter-directed transcription which are executed by its much larger and more familiar multi-subunit cousins. Thus it is an ideal system in which to study RNA polymerase structure and the mechanism of transcription. T7 RNA polymerase is simple enough to be approached from a biochemical perspective and to have its mechanism characterized quantitatively in terms of rate constants, substrate affinities, and allosteric modulation. It is also a DNA directed RNA polymerase subject to transcriptional regulation so an understanding of its mechanism is helpful in gaining a deeper understanding of how more complex RNA polymerases work. It is becoming increasingly apparent that an understanding of RNA polymerase mechanism at such a level will be necessary for an understanding of the regulation of gene expression as the qualitative description of transcription factors and regulation pathways is giving way to a more quantitative description of regulation in terms of formal allostery and the modulation of RNA polymerase initiation, elongation, and termination rates.

Selected Publications

Bonner, G., Patra, D., Lafer, E.M. and R. Sousa. Mutations in T7 RNA polymerase which support the proposal for a common polymerase active site structure. EMBO J. 11:3767-3775, 1992.

Sousa, R., Chung, Y.J., Rose, J. and Wang, B.C. Structure of T7 RNA polymerase at 3.3 Å resolution. Nature 364:593-599, 1993.

Bonner, G., Lafer, E.M. and Sousa, R. The thumb subdomain of T7 RNAP stabilizes the ternary complex against dissociation. J. Biol. Chem. 269:25129-25136, 1994.

Sousa, R., Rose, J. and Wang, B.C. The thumb's knuckle: Flexibility in the thumb subdomain of T7 RNA polymerase is revealed by the structure of a chimeric T7/T3 RNA polymerase. J. Mol. Biol. 244:6-12, 1994.

Sousa, R. and Padilla, R. A mutant T7 RNA polymerase as a DNA polymerase. EMBO J. 14:4609-4621, 1995.

Judy M. Teale, Ph.D.

Professor, Department of Microbiology
Ph.D., University of Virginia

The goal of our laboratory is to understand B lymphocyte differentiation. B cells produce antibodies as a result of antigenic challenge, e.g., the introduction of infectious organisms. The body contains a vast array of B lymphocytes, each of which can respond to a different antigen and produce antibodies of unique specificities. In this way, the body is protected against a universe of antigens.

One of the projects of the laboratory is to determine how such a diverse B cell immune repertoire is generated. Diversity is created, in part, by the recombination of small gene segments that encode the variable region or antigen binding site of the antibody molecule. One fascinating aspect in the generation of the repertoire, is that B cell specificities develop in a predictable temporal order. These early specificities may be important in the defense of the fetus and neonate against infectious organisms. Another important finding is that there is a difference in variable region gene expression in neonates vs adults. Our data suggest that these differences may relate to distinct B cell subsets. Using cellular and molecular approaches, our studies will define distinct B cell progenitors, determine the role of the microenvironment, and delineate genetic mechanisms involved in the generation of a diverse immune repertoire.

Another form of B cell differentiation is the production of various antibody classes or isotypes. Different antibody classes result from the ability of the progeny of individual, antigen stimulated B cells to express the same variable region on different constant regions, a process called the isotype switch. Since each isotype carries out distinct functions in the body, the particular isotypes that are produced during infection and in many disease states can often determine the course of infection or severity of disease. We are studying the isotype switch using a parasite infection model in which the infected animals selectively produce IgG1 and IgE. We have found that complex interactions between host and parasites, involving molecules shed or secreted by the organism, influence antibody induction. We are currently characterizing the required mediators and defining the precise mechanisms associated with the host immune response.

Selected Publications

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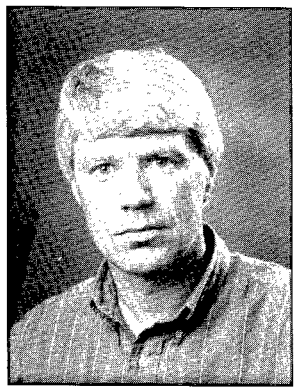
Ernani, F. and Teale, J. Release of stress proteins from Mesocestoides corti is a brefeldin A inhibitable process. Evidence for active export of stress proteins. Infect. Immun. 61:2596, 1993.

Turaga, P., Berton, M. and Teale, J. Frequency of B cells expressing germline gamma1 transcripts upon IL4 induction. J. Immunol. 151:1383, 1993.

Bangs, L., Sanz, I. and Teale, J. Comparison of D,JH and junctional diversity in the fetal, adult, and aged B cell repertoire. J. Immunol. 146:1996, 1991.

Alan E. Tomkinson, Ph.D.

Assistant Professor, Institute of Biotechnology
Ph.D., The University of Newcastle upon Tyne, England



My laboratory is interested in the network of pathways which maintain genomic stability in eukaryotes. Defects in these pathways can lead to genetic instability, a decrease in cell viability and an increase in the frequency of carcinogenesis.

We have chosen to study eukaryotic DNA joining enzymes, DNA ligases, since mutations in the DNA ligase genes of prokaryotes and lower eukaryotes cause genetic instability. Mammalian cells contain at least three biochemically distinct DNA ligases. One of these enzymes, DNA ligase I, is required for DNA replication and also functions in DNA repair. Recently, we have isolated cDNAs encoding mouse and human DNA ligase III. This gene is ubiquitously expressed at low levels except in the testes, which contain about 10-fold higher levels of DNA ligase III mRNA. Within the testes, the highest levels of DNA ligase III expression are found in cells undergoing meiotic recombination, suggesting that DNA ligase III joins DNA strand breaks introduced as a consequence of meiotic recombination. The cellular functions of DNA ligase III in somatic and germ cells are being investigated.

The ultraviolet component of sunlight is a major environmental mutagen that causes skin cancer. This type of DNA damage is normally repaired by a nucleotide excision repair pathway that is conserved amongst eukaryotes. Patients with the inherited cancer-prone disease, xeroderma pigmentosum, are specifically defective in this repair pathway. Recently, we have characterized an endonuclease activity from yeast that is required for DNA lesion removal. Since this endonuclease has no apparent affinity for DNA damage, we are searching for interactions between this enzyme and other components of the repair pathway that will confer DNA damage-specificity on the endonuclease. The goals of these studies are to elucidate the molecular mechanisms of nucleotide excision repair.

Selected publications

Tomkinson, A.E., Bardwell, A.J., Bardwell, L., Tappe, N. and Friedberg, E.C. Yeast DNA repair and recombination proteins Rad1 and Rad10 constitute a single-stranded DNA endonuclease activity. Nature 362:860-862, 1993.

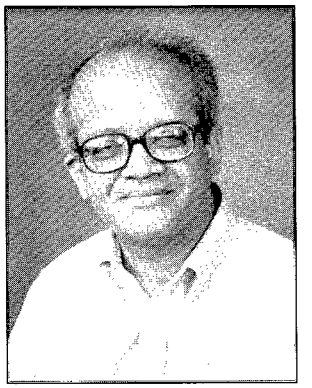
Bardwell, A.J., Bardwell, L., Tomkinson, A.E. and Friedberg, E.C. Specific cleavage of model recombination and repair substrates by the yeast Rad1/Rad10 endonuclease. Science 265:2082-2085, 1994.

Wang, Y.-C.J., Burkhart, W.A., Mackey, Z.B., Moyer, M.B., Ramos, W., Husain, I., Chen, J., Besterman, J.M. and Tomkinson, A.E. Mammalian DNA ligase II is highly homologous with Vaccinia DNA ligase. J. of Biol. Chem. 269:31923-31928, 1994.

Chen, J., Tomkinson, A.E., Ramos, W., Mackey, Z.B., Danehower, S., Walter, C.A., Schultz, R.A., Bestermon, J.M. and Husain, J. Mammalian DNA ligase III: Molecular cloning, chromosomal localization and expression in spermatocytes undergoing meiotic recombination. Molec. Cell. Biol. 15:5412-5422, 1995.

Manjeri Venkatachalam, M.B., B.S.

Professor, Department of Pathology
M.B., B.S., Calcutta University, India



We are investigating the molecular mechanisms of cell death. Our research on the necrotic form of cell death is focused mainly on the newly discovered protective actions of glycine against a specific form of plasma membrane. Our studies have shown that glycine and a related set of structurally similar amino acids have overriding actions which prevent plasma membrane damage in ATP depleted cells. Ongoing research is directed at a molecular approach towards the identification of the protein target of glycine.

We are also studying the molecular basis of programmed cell death (apoptosis) under conditions of increased intracellular generation of free radicals. Cell death may occur as a consequence of ischemia/hypoxia not only through a form of generalized structural breakdown, termed necrosis, but also by the more recently recognized, energy dependent and specifically programmed pathway of apoptosis. It is thought that reoxygenation of cells injured by ischemia by the reflow of blood may result in increased formation of radicals and initiation of apoptosis. We have developed a novel model system to characterize the biochemistry and molecular pathology of apoptosis. We are studying the possible roles played by the proto-oncogene bcl-2, and interleukin converting enzyme (ICE), recently discovered to be a mediator of apoptosis in neuronal cells, in the MDCK model. In addition to this model of apoptosis, we are also following two other lines of investigation: (1) Programmed cell death in the P 19 embryonal carcinoma cell line, and (2) the molecular basis for plasma membrane damage in the necrotic form of cell death, and its prevention by glycine.

Selected Publications

Rennke, H.G., Olson, J.L. and Venkatachalam, M.A. Glomerular filtration of macromolecules. Normal mechanisms and the pathogenesis of proteinuria. Contrib. Nephrol. 24:30-41, 1981.

Venkatachalam, M.A., Patel, Y.J., Kreisberg, J.I. and Weinberg, J.M. Energy thresholds which determine membrane integrity and injury in a renal epithelial cell line (LLC-PK1). Relationships to phospholipid degradation and unesterified fatty acid accumulation. J. Clin. Invest. 81:745-758, 1988.

Weinberg, J.M., Davis, J.A., Roeser, N.F. and Venkatachalam, M.A. Role of increased cytosolic free calcium in the pathogenesis of rabbit proximal tubule cell injury and protection by glycine or acidosis. J. Clin. Invest. 87:581-590, 1991.

Venkatachalam, M.A. and Weinberg, J.M. Structural effects of intracellular amino acids during ATP depletion. In "Surviving hypoxia: Mechanisms of control and adaptation." PW Hochachka, PL Lutz, G van den Thillart (eds) CRC Press, Boca Raton, FL, pp. 473-493, 1993.

Abrams, J.R., Tapp, D.C. and Venkatachalam, M.A. Dietary influence and pathologic changes. In "Progressive nature of renal disease." NE Mitch (ed), Churchill Livingstone, New York, in press.

Daniel D. Von Hoff, M.D.

Professor, Cancer Therapy and Research Center
M.D., The University of Columbia

I am a medical oncologist with research interests in the preclinical and clinical development of human cancer therapy. One of the hallmarks of tumors in patients is heterogeneity in their behavior and in their response to currently available therapies. This heterogeneity, which is most likely genetically based, is the main reason we have so much difficulty in curing patients with cancer.

In the laboratory, I work on the mechanisms of development of this genomic heterogeneity, which we refer to as genome plasticity. More specifically, we have discovered that tumors taken directly from patients have extrachromosomal DNA similar to plasmids noted in bacteria. We have named these submicroscopic pieces of DNA "episomes." Episomes are circular and can polymerize to form microscopically visible double minutes. Both episomes and double minutes are of interest because they contain genes which must be important to the tumor cell, since they are maintained in an extrachromosomal site. We believe these episomes and double minutes are the reasons for tumor cell genome plasticity. Genes residing on extrachromosomal DNA include amplified copies of oncogenes, drug resistance genes, genes for growth factor receptors, and other genes associated with progression and resistance of patients' tumors.

Our laboratory effort is directed at developing methods for the elimination of extrachromosomal DNA from patients' tumor cells. We have succeeded in elimination of copies of c-*myc* from patients' ovarian cancer cells. We are now working on mechanisms for improving that elimination. In addition, we are harvesting extrachromosomal DNA to isolate new genes of importance in genomic plasticity. An important aspect of my laboratory is the cultivation of an environment for translational research, where bench discoveries are constantly being related and applied to medical problems in patients.

Selected Publications

Von Hoff, D.D., Waddelow, T., Forseth, B., Davidson, K., Scott, J. and Wahl, B. Hydroxyurea accelerates loss of extrachromosomally amplified genes from tumor cells. Cancer Research 51: 6273-6279, 1991.

Von Hoff, D.D., McGill, J.R., Forseth, B.J., Davidson, K.K., Bradley, T.P., Van Devanter, D.R. and Wahl, G.M. Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. Pro. Natl. Acad. Sci. USA 89:8165-8169, 1992.

Thiebaut, F., Hanauske, A-R. and Von Hoff, D.D. Evidence for binding of extrachromosomal DNA sequences to nuclear matrix proteins in muiltidrug-resistant KB-V1 cells. Federation of European Biochemical Societies 319:133-137, 1993.

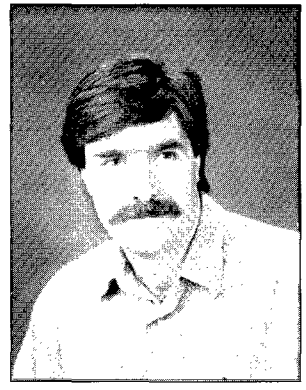
McGill, J.R., Beitzel, B.F., Nielsen, J.L., Walsh, J.T., Drabek, S.M., Meador, R.J. and Von Hoff, D.D. Double minutes are frequently found in ovarian carcinomas. Cancer Genet. Cytogenet. 71:125-131, 1993.

Eckhardt, S.G., Dai, A., Davidson, K.K., Forseth, BJ., Wahl, G.M. and Von Hoff, D.D. Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-myc. Proc. Natl. Acad. Sci. USA 91:6674-6678, 1994.

Bradford E. Windle, Ph.D.

Assistant Professor, Cancer Therapy and Research Center/
Department of Cellular and Structural Biology
Ph.D., The University of Maryland

My lab studies chromosome instability in mammalian cells that results from exposure of free ends due either to telomere loss or chromosome breakage. Recombination and repair mechanisms of this unstable DNA, particularly the process of new telomere addition, are being investigated. Telomerase is a reverse transcripting DNA polymerase that synthesizes the specific telomeric repeat sequence found at ends of mammalian chromosomes. The mechanism of action and regulation of telomerase is currently being investigated. The role of telomerase and telomeres in maintaining chromosome stability is a primary focus. Telomerase is an excellent target for anti-cancer therapy because it appears to be required for immortalization but is not expressed in normal somatic cells. My lab collaborates with others to develop telomerase inhibitors as tools for exploring chromosome stability and for future cancer therapeutics.



Selected Publications

Windle B.E., Draper B., Yin Y., O'Gorman S. and Wahl G.M. Chromosome breakage in gene amplification, generation of deletions and targeted integration. Genes and Development 5:160-174, 1991.

Windle, B.E. and Wahl, G.M. Molecular dissection of mammalian gene amplification: New mechanistic insights revealed by analyses of very early events. Solicited review, Mutation Research 276:199-224, 1992.

Wahl, G.M., Carroll, S. and Windle, B.E. Cytogenetic and Molecular Dynamics of Mammalian Gene Amplification: Evidence Supporting Chromosome Breakage as an Initiating Event. In: Gene Amplification in Mammalian Cells (R. Kellems, ed.), pp. 513-531, 1992.

Parra, I. and Windle, B.E. High Resolution Visual Mapping of Stretched DNA by Fluorescent Hybridization. Nature Genetics 5:17-21, 1993.

Windle, B.E., Silvas E. and Parra, I. High resolution microscopic mapping of DNA using multicolor fluorescent hybridization. Electrophoresis 16:273-278, 1995.

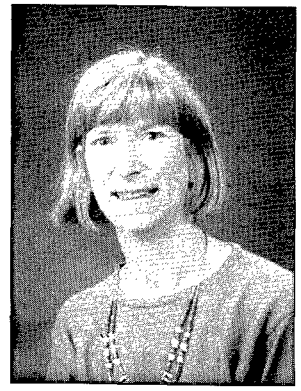
Jolene J. Windle, Ph.D.

Assistant Professor, Cancer Therapy and Research Center/Department of Cellular and Structural Biology
Ph.D., The Johns Hopkins University

The major focus of research in my laboratory is the investigation of the molecular basis of oncogenesis using transgenic mouse models. In particular, we are interested in the role of the retinoblastoma susceptibility (Rb) and p53 tumor suppressor genes in tumorigenesis in a variety of tumor types, including retinoblastoma and mammary adenocarcinoma.

To investigate the molecular basis of oncogenesis in retinoblastoma, we have created transgenic mice expressing a variety of DNA tumor virus oncoproteins specifically in photoreceptor precursors. These include SV40 T-antigen (Tag, which binds and inactivates both pRb and p53), and human papillomavirus E7 and E6 (which inactivate pRb or p53, respectively). Mice expressing Tag develop nonfocal, uniform retinoblastomas, indicating that Tag expression is sufficient to transform this cell type. In contrast, E7 expression results in dramatic retinal degeneration due to apoptotic cell death of all photoreceptor precursors. When E7 mice are bred into a p53-deficient background, retinoblastomas do arise, as expected. However, these tumors arise focally in an otherwise degenerated retina. These studies thus demonstrate that E7 expression can trigger both p53-independent and p53-dependent pathways of apoptosis. Our present studies involve the production of transgenic mice expressing various apoptosis-regulating genes studying their effect on both retinal degeneration and tumorigenesis.

A second project involves investigation of the role of p53 in apoptosis, drug responsiveness and genomic instability in mammary adenocarcinoma. We are interbreeding MMTV-ras and p53-/-mice to generate mice that develop mammary tumors in the background of a wild type or p53-deficient genotype. There is a growing body of literature suggesting that tumor responsiveness to a variety of chemotherapeutic agents can be correlated to the ability of the tumor to undergo apoptosis, and that p53 is a major regulator of this apoptotic response. Thus, p53 status could be a significant factor in multidrug resistance. To address this experimentally, we are testing the ability of tumors of mice from each genotype to respond to various forms of chemotherapy, and assessing the extent of growth arrest and apoptosis.



Selected Publications

Bignon, Y-J., Chen, Y., Chang, C-Y., Riley, D.J., Windle, J.J., Mellon, P.L. and Lee, W-H. Expression of a retinoblastoma transgene results in dwarf mice. Genes & Development 7:1654-1662, 1993.

Howes, K.A., Lasudry, J.G.H., Albert, D.M. and Windle, J.J. Photoreceptor cell tumors in transgenic mice. Invest. Ophthal. Vis. Sci. 35:342-351, 1994.

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Douglas Yee, M.D.

Associate Professor, Department of Medicine
M.D., University of Chicago

My laboratory is interested in the regulation of cancer cell growth by the insulin-like growth factors (IGFs). The IGFs are important regulators of the normal growth and development for many organ systems. We have previously shown that the IGFs are also mitogens for many cancer cell types. Most of the mitogenic effects of the IGFs are mediated through a specific cell surface receptor (the type I IGF receptor). Furthermore, high affinity binding proteins (IGFBPs) influence ligand receptor interactions. Taken together, these observations suggest that the IGFs could regulate breast cancer growth and that interruption of IGF mediated growth pathways could be used as a therapeutic strategy.

To test these hypotheses, we are evaluating the IGF system in breast cancer two ways. First, we are studying the expression of IGF system components in primary breast cancer specimens. We have discovered that certain components correspond with progression-free survival. For example, high levels of IGFBP-4 identify patients with relatively good prognosis, while levels of insulin receptor substrate-1 (IRS-1) identify patients with poor prognosis. These studies suggest a role for the IGFs in the growth regulation of primary breast cancer.

Our second approach uses tissue culture and animal model systems to develop methods to inhibit IGF signalling pathways. We have used a recombinant IGF binding protein (IGFBP-1) to inhibit *in vitro* breast and prostate cancer growth. Recently, we have used IGFBP-1 conjugated to polyethylene glycol to improve the pharmacokinetic characteristics of the recombinant protein. Preliminary studies demonstrate that this compound is an effective inhibitor of breast cancer growth in an animal model. We have also identified dominant negative type I IGF receptor constructs that inhibit the tyrosine kinase activity of the type I IGF receptor. We are studying inducible expression systems to develop these constructs for potential *in vivo* gene transfer studies.

Selected Publications

Yee, D., Jackson, J.G., Kozelsky, T.W. and Figueroa, J.A. Insulin-like growth factor binding protein-1 (IGFBP-1) expression inhibits IGF-I action in MCF-7 breast cancer cells. Cell Growth and Differentiation 5:73-77, 1994.

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Yee, D., Van Den Berg, C., Kozelsky, T.W., Kuhn, J.G. and Cox, G.N. Pharmacokinetic profile of recombinant human insulin-like growth factor binding protein-1 in athymic mice. Biomedicine & Pharmacotherapy, in press.

Figueroa, J.A., Lee, A.V., Jackson, J.G. and Yee, D. Proliferation of cultured human prostate cancer cells is inhibited by insulin-like growth factor binding protein-1: evidence for an IGF-II autocrine loop. Journal of Clinical Endocrinology and Metabolism, in press.

Notes

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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Reports to be changed to "Approved for public release; distribution unlimited"

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DAMD17-94-J-4437	ADB258772
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DAMD17-94-J-4437	ADB233377
DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900

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