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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

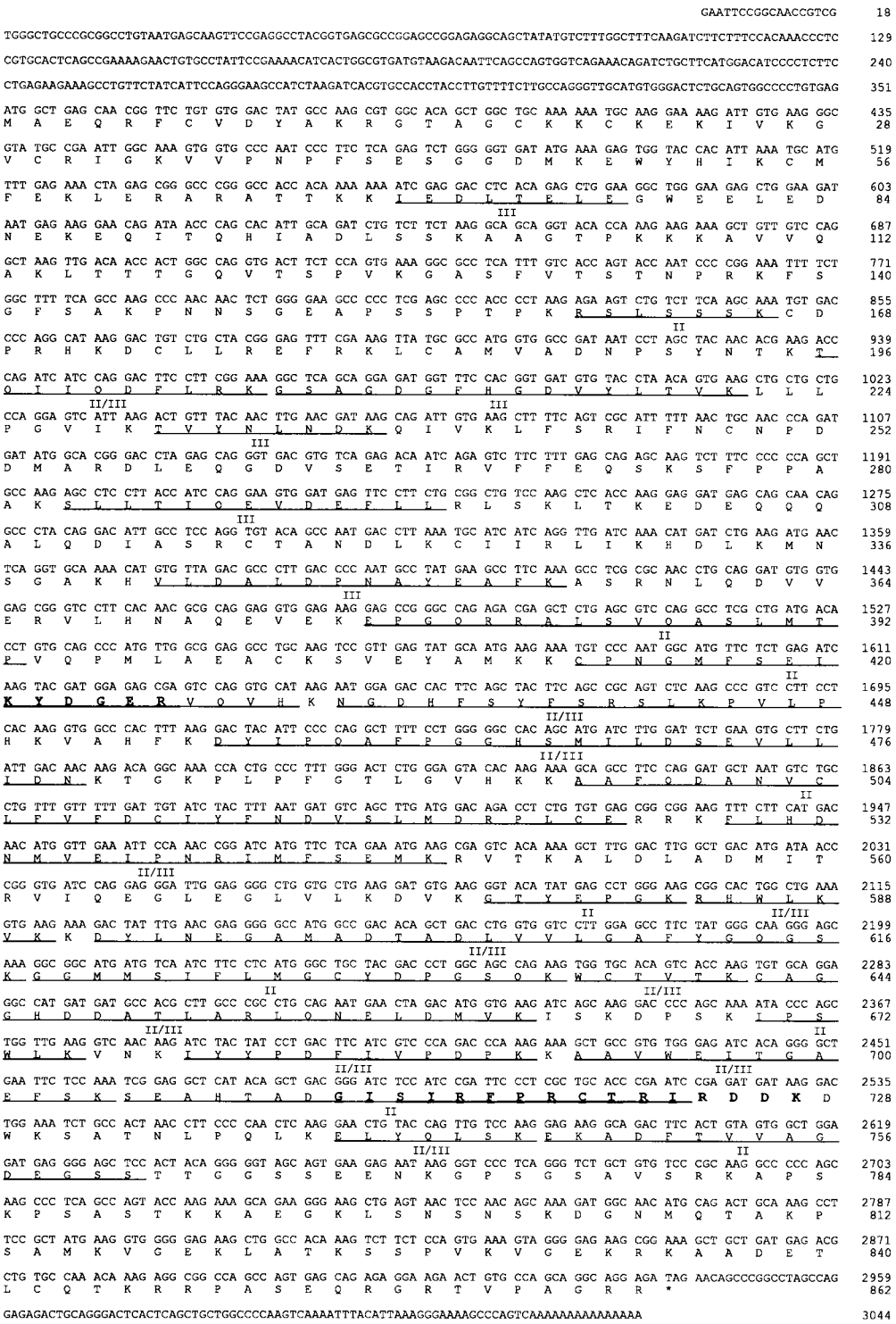

FIG. 1. Nucleotide sequence of human DNA ligase III cDNA. The predicted amino acid sequence is presented below the nucleotide sequence. Nucleotide and amino acid residues are numbered on the right. Regions homologous to the peptides from bovine DNA ligases II (indicated by II) and III (indicated by III) 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).

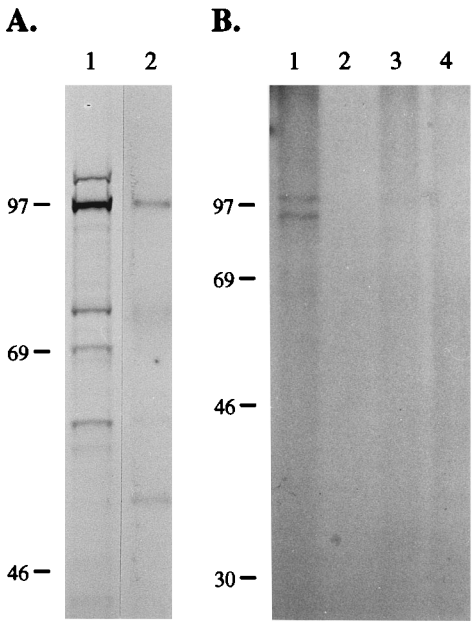

FIG. 2. In vitro translation of human DNA ligase III cDNA; reactivity of in vitro-translated DNA ligase III. (A) In vitro transcription and translation of full-length human DNA ligase III cDNA were performed as described in Materials and Methods. Lanes: 1, ^{35}S-labeled in vitro-translated polypeptides (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

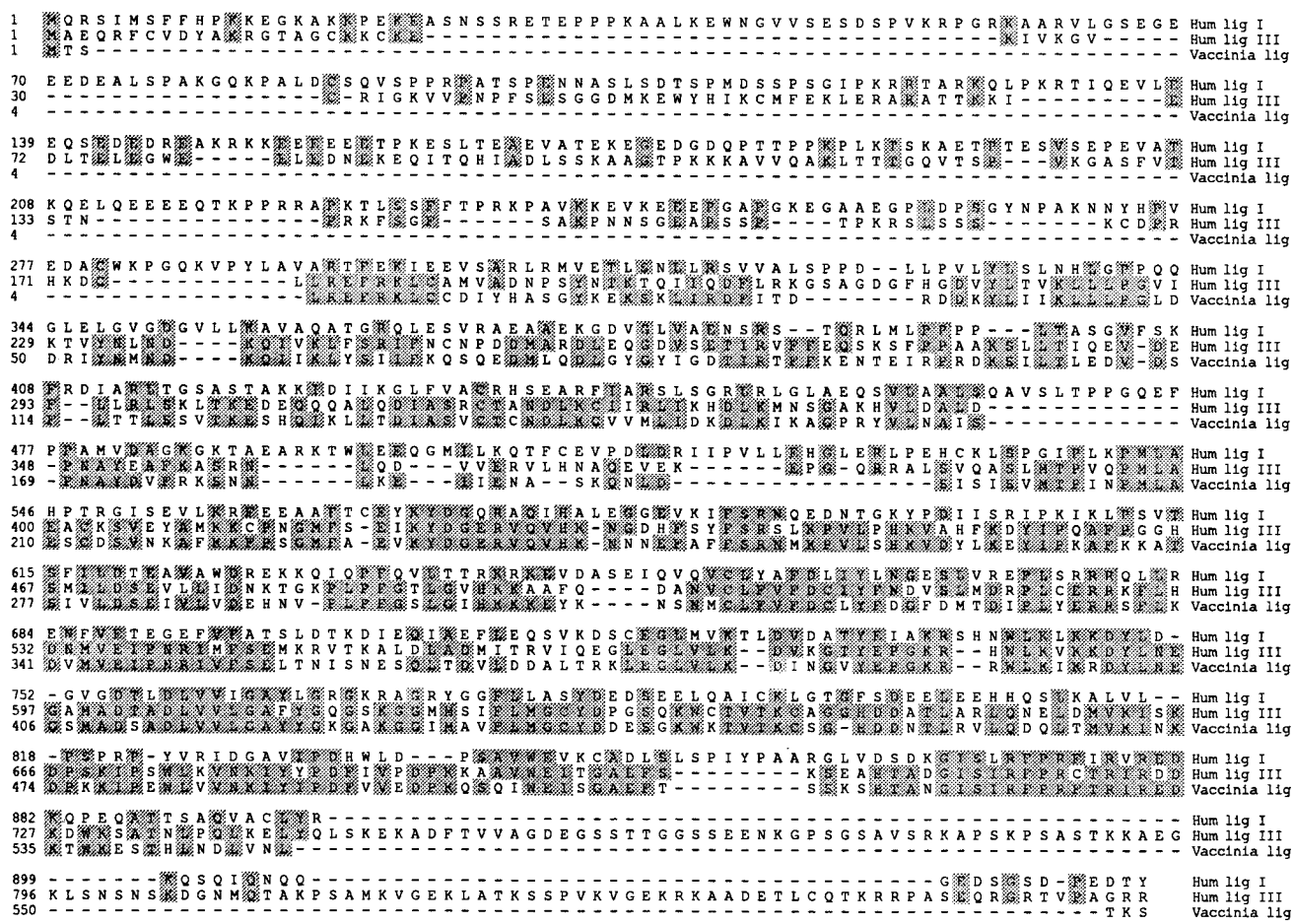

FIG. 3. Alignment of human DNA ligase (hum lig) I, human DNA ligase III, and vaccinia virus DNA ligase (vaccinia lig) protein sequences. The amino acid sequences of human DNA ligase I (4), vaccinia virus DNA ligase (48), and human DNA ligase III were aligned by using the DNAStar MegAlign program. Identical 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.

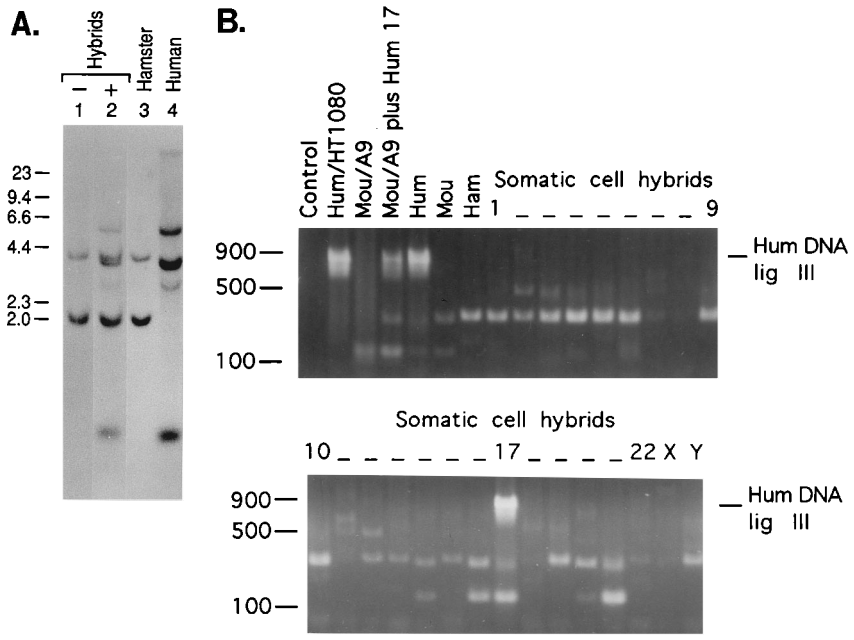

FIG. 4. Chromosomal localization of the DNA ligase III gene. (A) Southern analysis of *Pst*I-digested genomic DNAs (8 μg) from human, hamster, and hybrid somatic cells (BIOS Laboratory, Inc.) with an 810-bp *Eco*RI-*Xho*I DNA ligase III cDNA fragment as described in Materials and Methods. Only a selected 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).

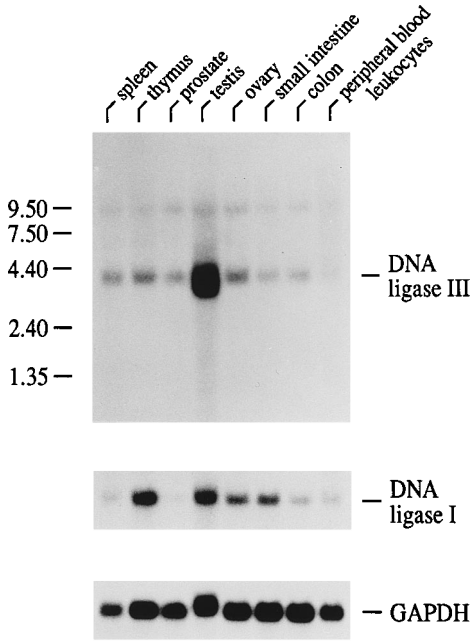

FIG. 5. Tissue and cell distribution of DNA ligase I and DNA ligase III mRNAs. A Northern blot containing ~2 μg of poly(A)$^+$ RNAs from various human tissues and cells was sequentially hybridized with a 400-bp human DNA ligase III cDNA fragment (nucleotides 2453 to 2843), a 1.4-kb DNA ligase I cDNA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

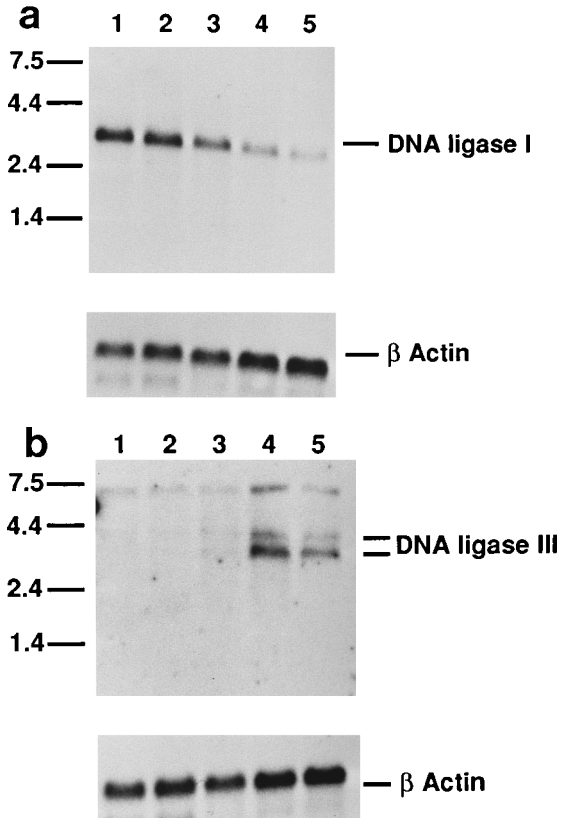

FIG. 7. Steady-state levels of DNA ligase I and DNA ligase III mRNAs in developing mouse testes. Duplicate poly(A)$^+$ samples (2 μg) from the testes of 5-day-old (lane 1), 8-day-old (lane 2), 15-day-old (lane 3), 25-day-old (lane 4), and 60-day-old (adult) (lane 5) mice were electrophoresed through 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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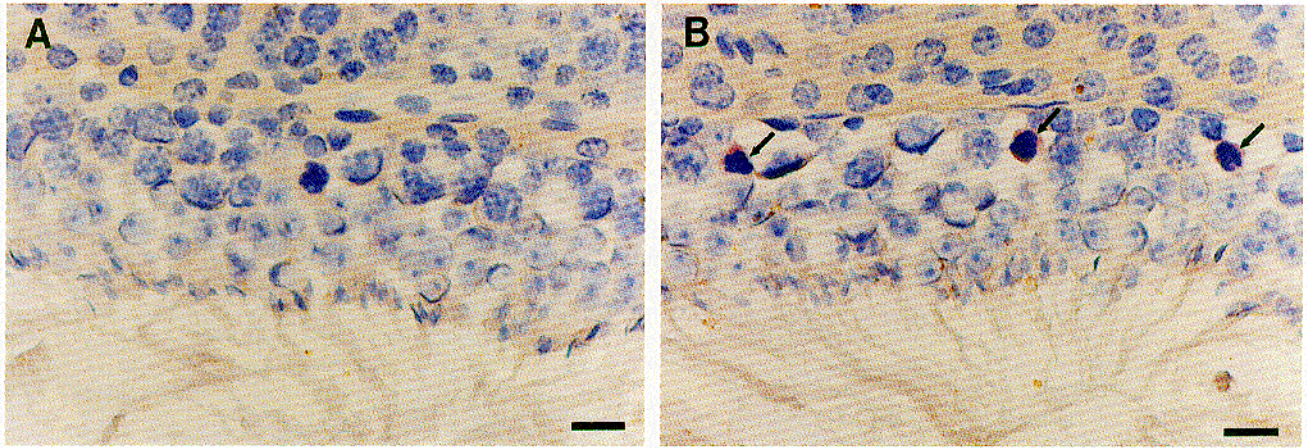
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