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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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Three mammalian genes encoding DNA ligases have been identified. However, the role 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 expressed only 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- to 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.

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 (4, 10, 45). Genetic and biochemical studies on the product of the *LIG1* gene indicate that this enzyme functions to join Okazaki fragments during DNA replication (4, 5, 30, 36, 42, 46). 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 extracts implicate DNA ligase I in DNA base excision repair (5, 20, 24, 30, 38). The recent characterization of an interaction between DNA polymerase β, which is essential for base excision repair of alkylation damage in mammalian cells (35), 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 (29).

The *LIG3* and *LIG4* genes encode polypeptides that have similar electrophoretic mobilities in denaturing polyacrylamide gels (45). 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 (8, 9, 45). Human *XRCC1* was cloned by its ability to complement the hypersensitivity of the Chinese hamster ovary cell line EM9 to DNA-alkylating agents (39, 40). 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 (8, 9, 25, 39, 40). At present, very little is 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 (10). 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 (10). Furthermore, expression of the *XRCC1* gene, whose product interacts with DNA ligase III (8, 9, 45), is also

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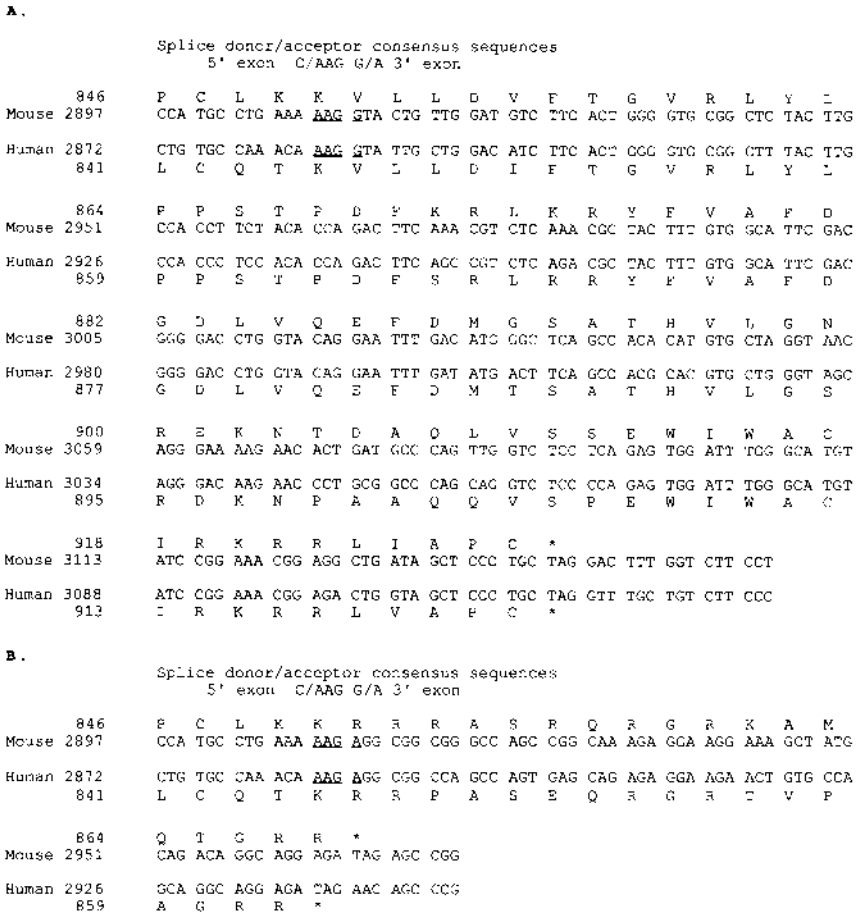

FIG. 1. Alignment of the carboxy-terminal amino acid sequences encoded by the different 3′ nucleotide sequences of mammalian DNA ligase III cDNAs. Human DNA ligase III cDNA sequences that differ at their 3′ ends have been reported previously (10, 45). Nucleotide and amino acid residues are numbered on 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.

elevated in both pachytene spermatocytes and round spermatids (43, 44).

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 occurs 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 (10, 45). These cDNAs have different 3′ ends and encode different-size polypeptides with distinct C termini (Fig. 1). Within the identical regions of the HeLa and testis DNA ligase III cDNAs, there is an internal *Eco*RI site at nucleotides 2452 to 2457 in the testis cDNA (10). The nucleotide sequences of the HeLa and testis cDNAs diverge 435 nucleotides 3′ of the *Eco*RI site. Using the 600-bp sequence at the 3′ end of the testis cDNA (*Eco*RI-*Xba*I fragment) as a probe (10), we isolated a 1.1-kb cDNA fragment from a human liver cDNA library (Stratagene). The DNA sequence of this fragment is identical to that of the 3′ end of the HeLa DNA ligase III cDNA (45). 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 *Eco*RI-*Xba*I fragment of the testis DNA ligase III cDNA with the 1.1-kb *Eco*RI 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) by using human DNA ligase III cDNA as a probe (10). A full-length cDNA (3,080 bp), which contains an internal *Eco*RI 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 (10). This form of DNA ligase III has been designated DNA ligase III-β.

Using oligonucleotides GATGAGACGCTGTGCCAAA and GGAAGACA GCAAACCTAGC, which correspond to nucleotides 2863 to 2881 and the complement of nucleotides 3134 to 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, we amplified a 251-bp fragment by PCR (33). The PCR mixture (100 μl) contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate, 1 μM each oligonucleotide, 10 ng of cDNA template, and 2.5 U 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, we isolated a 1.3-kb cDNA from the mouse testis cDNA library. The DNA sequence at one end of this cDNA is identical to that 3′ of the *Eco*RI 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) (45). A mouse DNA ligase

III-α cDNA (3,832 bp), which was constructed by replacing the 3′ 600 bp of sequence from the *Eco*RI site with the 1.3-kb fragment, encodes a polypeptide consisting of 927 amino acids that has a calculated molecular weight of 103,000.

DNA sequencing and sequence analysis. Double-stranded DNA sequencing was carried out by the dideoxy-chain termination method with Sequenase and synthetic sequencing primers as described previously (10). DNA sequences were aligned by using Seqman and translated by using Editseq (DNAStar). Nucleotide and amino acid sequence homologies were aligned with Align (DNAStar).

Cloning and analysis of the mouse DNA ligase III gene. A 0.9-kb *Pst*I fragment (nucleotides 1448 to 2333) from mouse DNA ligase III cDNA was used as a probe to screen a mouse 129/J genomic library (Stratagene) by a standard protocol (34). A single phage containing an 18-kb genomic fragment was isolated and further analyzed by restriction enzyme mapping and Southern blotting (34). 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 PCR. Using oligonucleotides ACTGTTGGATGTCTTCACTGGG and AAAGACAAAGCTAGCACCCGGA, which correspond to nucleotides 2914 to 2935 and the complement of nucleotides 3349 to 3326 of the 3,832-bp mouse DNA ligase III-α cDNA, respectively (Fig. 1B), and the 3,832-bp mouse DNA ligase III-α cDNA as a template, we amplified a 425-bp fragment by PCR. The PCR conditions were as described above except that the annealing step was carried out at 62°C. Using oligonucleotides CAGCCGGCAAAGAGGAAGGA and TTTTTGCTGCACCCCACCGC, which correspond to nucleotides 2923 to 2942 and the complement of nucleotides 3073 to 3052 of the 3,080-bp mouse DNA ligase III-β cDNA, respectively (Fig. 1B), and the 3,080-bp mouse DNA ligase III-β cDNA as a template, we amplified a 150-bp fragment by 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 each deoxynucleoside triphosphate, 1 μM each oligonucleotide, 40 ng of cDNA template, and 2.5 U 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 used to obtain enriched populations of late pachytene spermatocytes, round spermatids, and residual bodies from the testes of adult male mice (31). Enriched populations of Sertoli cells and spermatogonia (types A and B) were isolated from testes of 8-day-old mice, while enriched populations of preleptotene, leptotene plus zygotene, and early pachytene spermatocytes were isolated from 17- to 18-day-old mice by using a modified StaPut gradient system (6). 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 (43). Total RNA was isolated from mouse tissues and purified germ cell populations by cesium chloride centrifugation after lysis in guanidinium isothiocyanate (14). 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 hybridized with a labeled DNA probe (10^9 cpm/μg) and then washed as described previously (10). Hybridizing bands were detected by autoradiography and 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% sodium dodecyl sulfate (SDS) at 90°C.

Reverse transcription-PCR (RT-PCR). Total RNA (2 μg) or poly(A)$^+$ RNA (0.2 μg) was used as the template for reverse transcription by avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) under the conditions suggested by the manufacturer. Aliquots (2 μl) that correspond to 40 ng of total RNA or 4 ng of poly(A)$^+$ RNA were used as templates for amplification by PCR of sequences unique to the α and β species of mouse DNA ligase III cDNA. These reactions 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 PhosphorImager (Molecular Dynamics) analysis.

The 30-cycle amplification used in the experiments described above 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, amplified only 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 (32) 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 to 700 was also subcloned in frame into the same vector. After transformation into *Escherichia coli* TG1, transformants were grown in Terrific broth medium with 0.1 mg of ampicillin per ml at 37°C. When the optical density at 600 nm of the culture reached 0.6, isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for 5 h at room temperature.

Preparation of DNA ligase III affinity resins. *E. coli* cells harboring plasmids that direct the expression of GST-DNA ligase III fusion proteins and GST were grown as described above. Cells (500-ml culture) were harvested and resuspended in 5 ml of buffer A (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.4 μg of aprotinin per ml, 0.5 μg each of chymostatin and leupeptin per ml, 0.7 mg of pepstatin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) and lysed by sonication. After clarification of the lysate by centrifugation, soluble proteins were mixed gently at 4°C for 1 h with 350 μl of glutathione-Sepharose 4B beads (Pharmacia Biotech) that had been equilibrated with buffer A. The beads were washed three times with buffer A and then stored on ice as a 50% slurry in buffer A. 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 (22), polypeptides were detected by staining with Coomassie blue. For each affinity bead, the major bound polypeptide corresponded to the expected size of GST or the GST-DNA ligase III fusion protein. Similar quantities of the GST-DNA ligase III fusion proteins and GST were bound to an equivalent volume of glutathione-Sepharose 4B beads. GST-DNA ligase III fusion proteins were eluted from the glutathione beads with 5 mM glutathione and incubated with [α-^{32}P]ATP. After separation by denaturing gel electrophoresis (22), labeled DNA ligase-adenylate intermediates were detected by autoradiography (41).

Purification of recombinant XRCC1 protein. Recombinant XRCC1 protein with a polyhistidine sequence at the C terminus (85 kDa) was purified from *E. coli* as described by Caldecott et al. (9).

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 previously (26). Recombinant XRCC1 protein was labeled and then incubated with the nitrocellulose membrane as described by Wei et al. (45).

Interaction between labeled XRCC1 and DNA ligase III affinity resins. XRCC1 protein was labeled both by phosphorylation (45) and by coupled in vitro transcription and translation. Glutathione affinity beads (30 μl) that had been preequilibrated with buffer B (50 mM HEPES [pH 7.7], 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 2% dried milk) were incubated with phosphorylated XRCC1 (0.1 μg, 210,000 cpm) in a final volume of 100 μl for 30 min at room temperature. After washing with buffer B minus the dried milk, SDS sample buffer (20 μl) was added to the beads, which were then incubated at 70°C for 10 min to remove non-covalently attached proteins from the beads. Labeled polypeptides were separated by denaturing gel electrophoresis, detected by autoradiography, and quantitated by scanning of the X-ray film with a laser densitometer (Molecular Dynamics).

For in vitro transcription and translation, an *Eco*RI fragment containing *XRCC1* cDNA was subcloned from pcD2EHX (8) into pBluescript KS (Stratagene). This XRCC1 polypeptide, which has an amino-terminal polyhistidine sequence, was synthesized by coupled transcription and translation (Promega). ^{35}S-labeled XRCC1 (90 kDa) was partially purified by ammonium sulfate precipitation (3) and resuspended in buffer C (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 5 mM EDTA, 0.1% Nonidet P-40). The glutathione affinity beads (30 μl) were washed 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 C. SDS sample buffer (20 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).

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 2-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.

Immunofluorescence. Mouse NIH 3T3 fibroblasts were grown in Dulbecco modified Eagle medium (GibcoBRL) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml on 12-mm-diameter cover glasses. Metaphase chromosome spreads were prepared by using the modification of Freshley (12) and dropped on clean glass slides. Late pachytene spermatocytes purified from adult mouse testis were attached to poly-L-lysine-coated glass slides. Microspreads of pachytene spermatocytes were prepared by the method of Speed (37). Attached cells were rinsed twice with phosphate-buffered saline, fixed for 3 min in ice-cold methanol, and then permeabilized by immersion in a solution of 0.25% Triton X-100 in phosphate-buffered saline for 3 min at room temperature. Incubations with the preimmune or immune sera and the fluorescein-conjugated secondary antibody (Kirkegaard & Perry) were performed as described previously (2). Slides were stained with

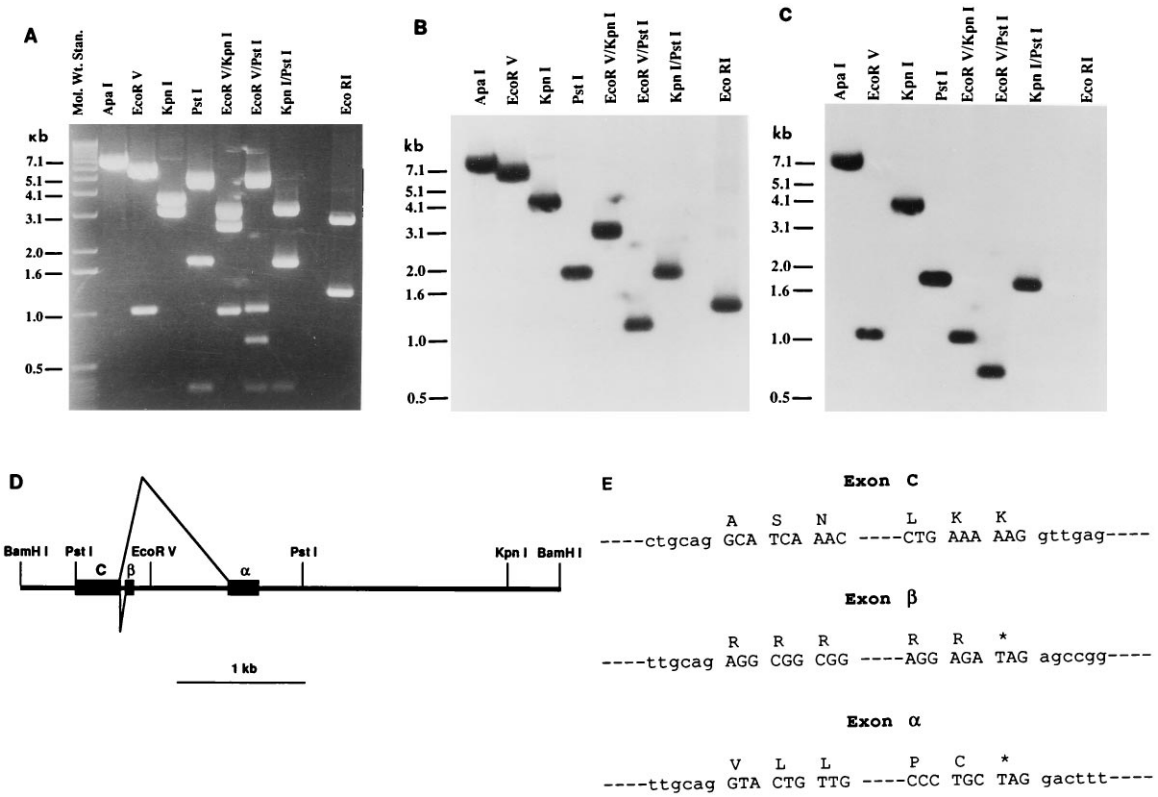

FIG. 2. Structural organization and restriction map at the 3′ end of the mouse *LIG3* gene. A genomic fragment containing the 3′ end of the mouse *LIG3* gene was isolated as described in Materials and Methods. A 4.2-kb *Bam*HI fragment of genomic DNA was subcloned into pBluescript KS, and the resultant plasmid was digested with *Apa*I, *Eco*RV, *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 *Eco*RI. (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. Mol. Wt. Stan., molecular weight standards. (B) The 425-bp probe specific for mouse DNA ligase III-α cDNA. (C) The 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 boxed region C. Boxed region β corresponds to the unique coding sequence at the 3′ end of DNA ligase III-β cDNA, and 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.

4′,6-diamino-2-phenolindole propidium iodide (DAPI; 1 μg/ml) and then examined on a Zeiss fluorescence microscope (magnification, ×1,250). Digital photographs were obtained with a Hamamatsu Photonics camera.

Nucleotide sequence accession numbers. The mouse DNA ligase III-β cDNA sequence has been deposited in GenBank under accession number U66057. The mouse DNA ligase III-α DNA sequence has been deposited in GenBank under accession number U66058.

RESULTS

Two forms of mammalian DNA ligase III cDNA. Human cDNAs encoding DNA ligase III have recently been reported by two different groups (10, 45). 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 sizes of 3.4 and 3.6 kb have been observed (10).

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 have about 90% amino acid identity overall, but the degree of identity is only 60% within the different C-terminal regions of 17 to 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 ligases III-α and III-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 to C). The order and arrangement of exons and introns at the 3′ end of the DNA ligase III gene shown in Fig. 2D were determined by restriction enzyme mapping, Southern blotting (Fig. 2A to C), 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 (28).

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 other tissues after longer exposures (data not shown), indicating that, as in humans (10), DNA ligase III is ubiquitously expressed at a low level except in the testes, where the steady-state levels of DNA ligase III mRNA are at least 10-fold higher than in other tissues and cells.

We have used a more sensitive technique, 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 of 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, 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 (10). 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 testes of 25-day-old animals that was about threefold 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 testes of 25-day-old animals, expression of this mRNA species in the testes of the younger animals was barely detectable (<5% of the steady-state levels in the testes of the 25-day-old animals).

In the testis of a 25-day-old mouse, the steady-state level of 3.4-kb DNA ligase III-β mRNA is about twofold 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 (6). If, as suggested, the steady-state levels of DNA ligase III mRNAs are

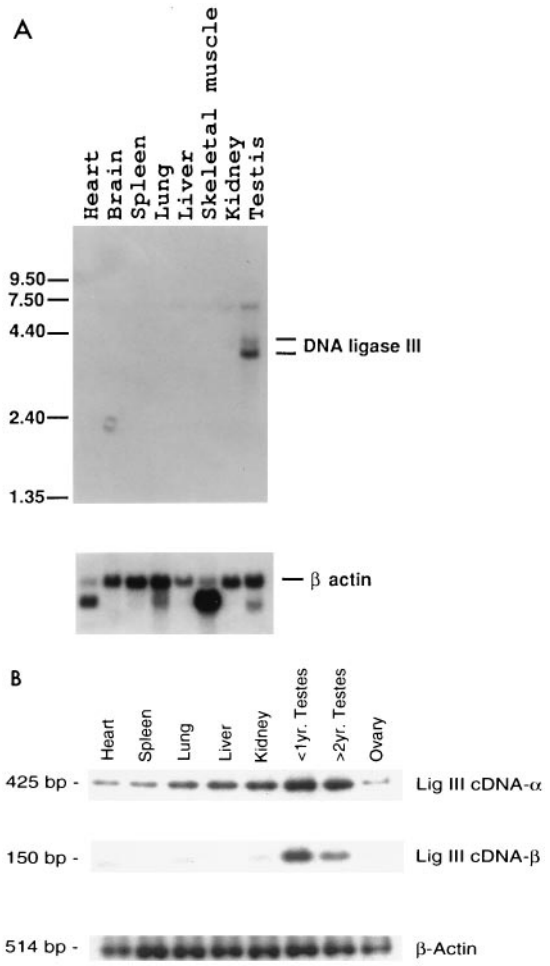

FIG. 3. Tissue and cell distribution of mouse DNA ligase III mRNAs. (A) A Northern blot containing ~2 μg of poly(A)$^+$ RNAs from various mouse tissues was sequentially hybridized with a 0.9-kb mouse DNA ligase III cDNA fragment (nucleotides 1448 to 2333) and a 2-kb human β-actin cDNA fragment. (B) Expression of the α and β forms of mouse DNA ligase (Lig) 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.

significantly higher in this cell type than in other cell types present in the testis, then poly(A)$^+$ RNA from pachytene spermatocytes should have a higher level of DNA ligase III mRNA than the same amount of poly(A)$^+$ RNA from whole testes 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 threefold higher in late pachytene spermatocytes than in 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 testes 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

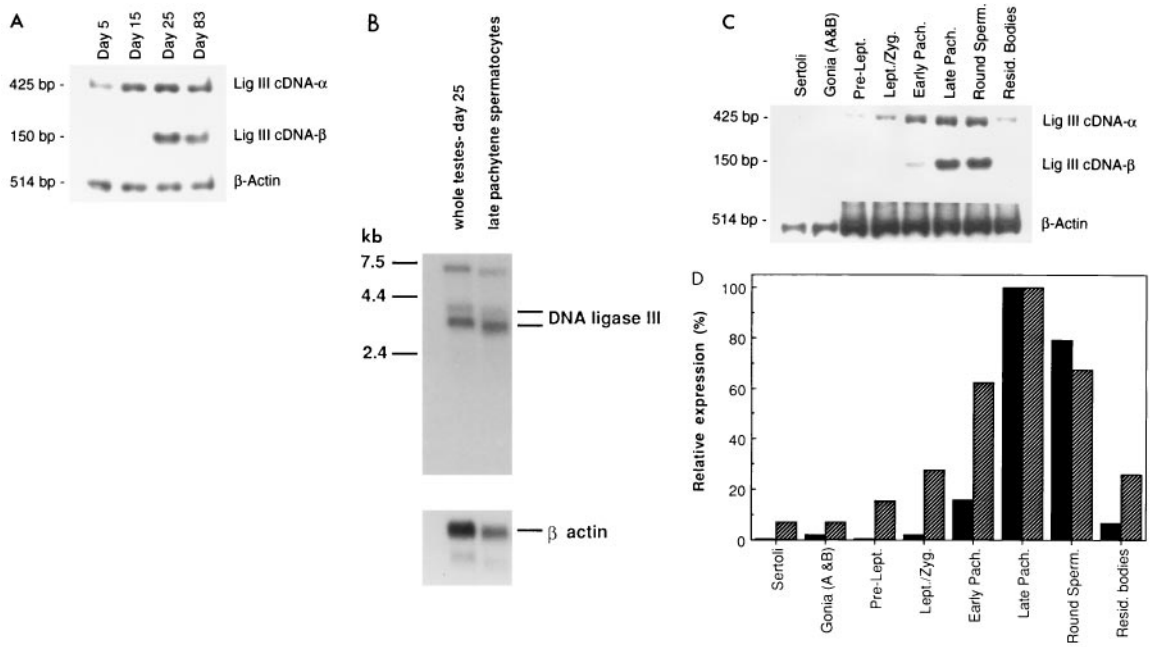

FIG. 4. Steady-state levels of DNA ligase III mRNAs during mouse testis development and in germ cell populations purified from mouse testis. (A) Expression of the α and β forms of mouse DNA ligase (Lig) III cDNA in the testes of 5-, 15-, 25-, and 83-day-old mice. RT-PCR was performed with poly(A)$^+$ RNA samples isolated from the testes of the 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 testes of 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 to 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-β cDNA, and the 514-bp PCR product amplified by primers specific for mouse β-actin are indicated. Gonia, spermatogonia; Lept., leptotene; Zyg., zygotene; pach., pachytene; sperm., spermatids; Resid., residual. (D) Graphic representation of the data shown in panel C. After quantitation of the PCR products by PhosphorImager 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.

Sertoli cells was initially detected in preleptotene 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 D). 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 D).

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 the 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 (10, 45).

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 that 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 (8, 9, 45), we have compared the binding of the α and β forms of DNA ligase III to XRCC1 by two different methods. In Fig. 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 (9, 45), XRCC1 formed a complex with the 103-kDa DNA ligase III-α. In contrast, no detectable complex was formed with DNA ligase III-β (Fig. 5B) or with GST protein (data not shown).

To confirm the results obtained by far-Western blotting, purified recombinant XRCC1 and in vitro-translated XRCC1 protein were incubated with GST and 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 and D). After the subtraction of background binding to GST beads, greater than 20-fold more ^{32}P-labeled recombinant XRCC1 bound to the glutathione beads with DNA ligase III-α as the ligand compared to glutathione beads with the same amount of either DNA ligase III-β or a GST-DNA ligase III fusion con-

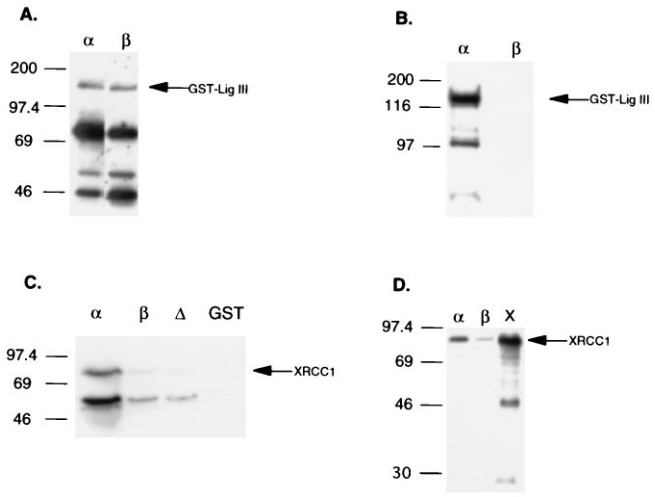

FIG. 5. Interaction of DNA ligase III-α and DNA ligase III-β with XRCC1. (A) GST-DNA ligase (Lig) III fusion proteins (10 ng) were adenylylated as described in Materials and Methods. After separation by denaturing gel electrophoresis, labeled polypeptides in the dried gel were detected by autoradiography. 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. Sizes on the left are indicated in kilodaltons. (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 previously (26, 45). 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 and GST-DNA ligase III fusion proteins bound to glutathione-Sepharose beads were incubated with ^{32}P-labeled, purified XRCC1 as described in Materials and Methods. Labeled polypeptides that bound to the beads were detected by autoradiography after separation by denaturing gel electrophoresis. (D) GST-DNA ligase III fusion proteins bound to glutathione-Sepharose beads were incubated with ^{35}S-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, β, Δ, and GST correspond to experiments with GST-DNA ligase III-α, GST-DNA ligase III-β, GST-DNA ligase III$_{1\text{-}700}$ (residues 1 to 700 of DNA ligase III), and GST beads, respectively. Lane X contains in vitro-translated XRCC1. The positions of ^{14}C-labeled molecular mass standards (Amersham) are shown on the left.

taining the amino-terminal 700 residues of DNA ligase III (common to both DNA ligase III-α and DNA ligase III-β) as the ligand (Fig. 5C). A labeled proteolytic fragment of XRCC1 exhibited the same binding pattern (Fig. 5C). In similar experiments with in vitro-translated XRCC1, preferential binding to the DNA ligase III-α affinity matrix was also observed (Fig. 5D). These results demonstrate that interaction between DNA ligase III and XRCC1 requires the C terminus of DNA ligase III-α.

Intracellular distribution of DNA ligase III in mitotic and meiotic cells. We have examined the subcellular distribution of DNA ligases I and III in unsynchronized, dividing populations of human and mouse cells by indirect immunofluorescence. In agreement with previous studies (23, 46), 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 (data not shown). In contrast, intense staining of the highly condensed chromatin present from metaphase to telophase was observed in both the human cells (data not shown) and mouse NIH 3T3 fibroblasts (Fig. 6A) with the DNA ligase III antiserum. No detectable staining of condensed chromatin was observed in similar experiments with preimmune serum and DNA ligase III antiserum that had been preincubated with purified DNA ligase III (data not shown). The association of DNA ligase III with the condensed chromatin present during the M phase of the cell cycle was confirmed by the staining of metaphase chromosomes with the DNA ligase III antiserum (Fig. 6B). 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).

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). To further address the relationship between DNA ligase III and the synaptonemal complex, spreads of pachytene spermatocytes were prepared. The similarity in staining patterns obtained with DAPI and the DNA ligase III antiserum is consistent with the association of DNA ligase III with condensed chromatin (Fig. 6D). This pattern can be superimposed on thread-like structures, visible by phase microscopy, that presumably correspond to the protein core of synaptonemal complexes (35). This finding suggests that DNA ligase III binds to the loops of chromatin that are attached to the protein core of the synaptonemal complex.

DISCUSSION

Several examples of differences in gene expression between somatic and germs cells, including different transcription initiation sites (16), differences in the length of the poly(A) tract (13, 18), alternative splicing (15), and the existence of homologous genes, one expressed only in somatic cells and the other expressed only in germ cells (19, 27), have been described. 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, which differs with respect to carboxy terminus and molecular mass from the ubiquitously expressed 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 its association with the human DNA repair protein XRCC1 (8, 9, 39, 40, 45). This finding 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 results 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 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 (8). 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

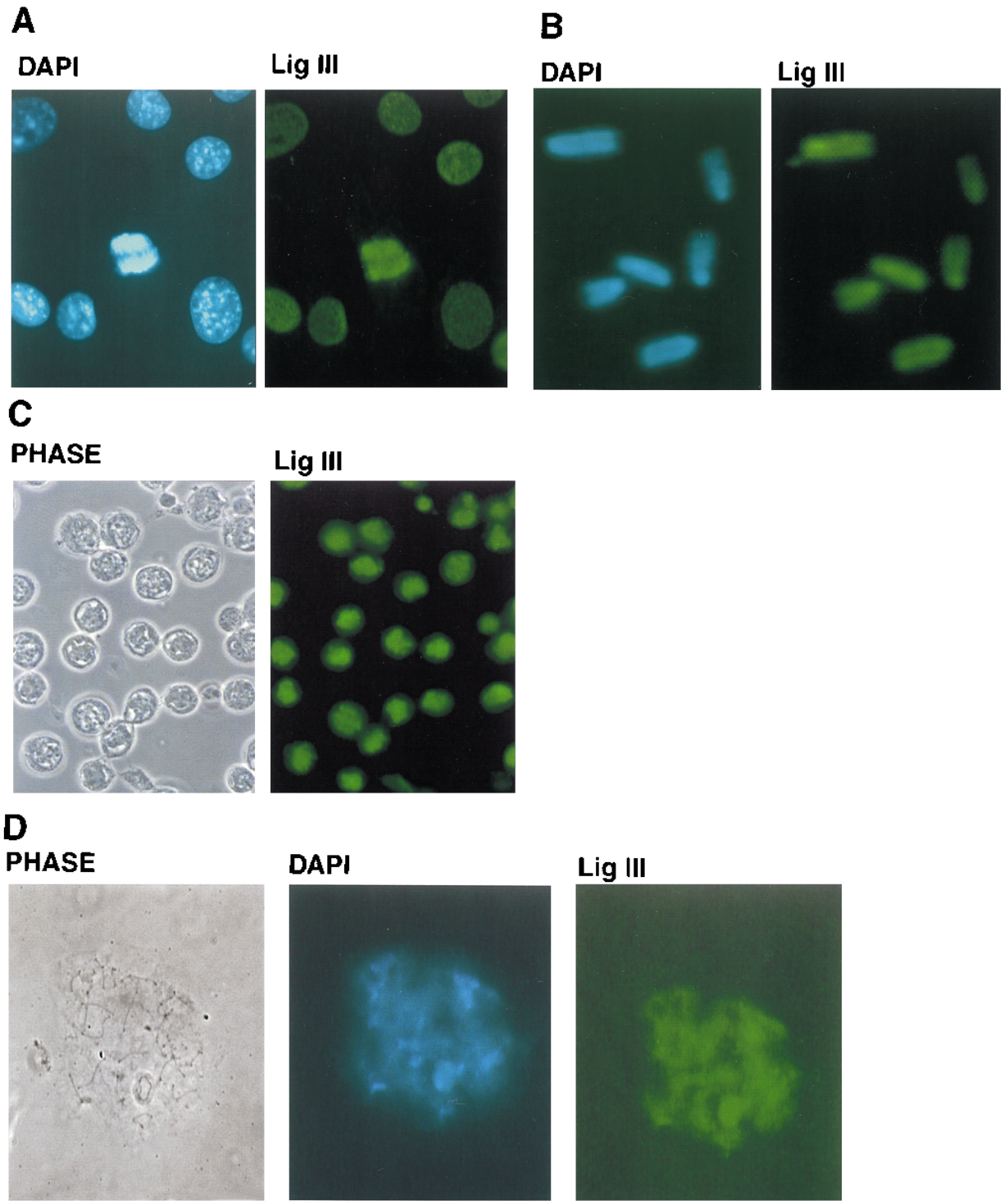

FIG. 6. Localization of DNA ligase III in somatic cells and in pachytene spermatocytes by indirect immunofluorescence. After fixation, samples were incubated with a mouse polyclonal antiserum specific for DNA ligase III (1:1,000 dilution) as described in Materials and Methods. Staining with DNA ligase III antibody was detected by indirect immunofluorescence using a fluorescein-conjugated secondary antibody (green fluorescence). (A) Mouse NIH 3T3 fibroblasts. Shown are DAPI staining detected by direct fluorescence and the same microscopic field analyzed by indirect immunofluorescence with the DNA ligase III antiserum (Lig III). (B) Mouse metaphase chromosomes. Shown are DAPI staining detected by direct fluorescence and the same microscopic field analyzed by indirect immunofluorescence with the DNA ligase III antiserum. (C) Mouse pachytene spermatocytes. Shown are cells visualized by phase-contrast microscopy (PHASE) and the same microscopic field analyzed by indirect immunofluorescence with the DNA ligase III antiserum. (D) Spread of mouse pachytene spermatocytes. Shown are a chromosome spread visualized by phase-contrast microscopy and the same microscopic field analyzed by DAPI staining and by indirect immunofluorescence with the DNA ligase III antiserum.

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 (43, 44), whose product interacts with DNA ligase III-α in somatic cells (8, 9, 45). This apparent coordinate 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 (21). 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 postmeiotic 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 (11). 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 binds to condensed chromatin in meiotic cells and thus becomes associated with the loops of chromatin that are attached 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 protein core of the synaptonemal complex, as has been observed for the eukaryotic recombination protein RAD51 (7, 17).

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 its 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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