Cloning of cDNA Encoding a Novel Mouse DNA Topoisomerase III (Topo IIIβ) Possessing Negatively Supercoiled DNA Relaxing Activity, Whose Message Is Highly Expressed in the Testis*

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We cloned cDNA encoding a novel mouse homologue of DNA topoisomerase III (mTOP3β). The nucleotide sequence contains an open reading frame of 863 amino acids, and the deduced molecular mass of the coded protein is 96.9 kDa. The overall sequence of mTOP3β has a 48 and 36% identity with mouse TOP3α at the nucleotide and amino acid level, respectively. DNA topoisomerase IIIβ was expressed using a baculovirus expression system and purified. The purified DNA topoisomerase IIIβ had activity to relax negatively supercoiled DNA. Relaxation of supercoiled DNA was partial at 37 °C and complete relaxation was observed at higher temperatures. mTOP3β mRNA was strongly expressed in the testis and relatively strongly in the brain. The levels of TOP3β mRNA in the testis increased slightly 14 days and considerably 17 days after birth, when the cells in the pachytene phase begin to appear and increase.

DNA topoisomerases are classified as type I or type II enzymes (1). Recently, DNA topoisomerase III belonging to the type IA subfamily, which includes Escherichia coli Topo I and Topo III and yeast Topo III (2–4), has been found in higher eukaryotic cells (5). The gene encoding yeast Topo III (TOP3) was identified in 1989 as a suppressor of mitotic recombination between repetitive sequences (4). The yeast top3 mutant has a slow growth phenotype as well as a hyperrecombination phenotype. It is speculated that Topo III has a significant role in the unlinking of parental strands at the final stage of DNA replication and/or in the dissociation of structures that could lead to recombination (6).

The slow growth and hyperrecombination phenotypes of yeast top3 mutants were suppressed by mutations in SGS1 gene whose product was shown to interact functionally and physically with Topo III (7). The sgs1 mutants also show a hyperrecombination phenotype (8). Thus it is conceivable that yeast Topo III acts in conjunction with Sgs1. Recently, human homologues of the SGS1 gene, BLM and WRN, have been cloned by positional cloning being the genes responsible for Bloom’s syndrome (BS) and Werner’s syndrome (WS), respectively (9, 10). BS patients suffer cancer predisposition, immunodeficiency, and male infertility. In the BS cells, the interchanges between homologous chromosomes are increased, and an abnormally large number of sister chromatid exchanges are present (11). WS is known as a disease that causes premature aging, and cells derived from patients show a reduced replicative life span and chromosome aberrations including deletion (10). In this context, mammalian Topo III is attracting attention.

A cDNA encoding human Topo III was cloned in 1996 (5). The gene disruption study of mouse TOP3 showed mammalian topo III was essential in early embryogenesis (12). Recently, a genomic sequence encoding a putative Topo III homologue was found within the human immunoglobulin λ gene locus (13). Thus, the Topo III encoded by the cloned cDNA and the putative Topo III in the genomic sequence were named by Li and Wang (12) as Topo IIIβ, respectively. However, nothing is known about Topo IIIβ.

In the course of cloning the mouse homologue to human TOP3α, we cloned cDNA encoding a mouse homologue to human Topo IIIβ. In this study, we examined both whether Topo IIIβ has DNA topoisomerase activity or not using a purified protein and also the expression of TOP3β mRNA in various mouse tissues and in the testis during postnatal development.

EXPERIMENTAL PROCEDURES

cDNA Cloning of TOP3β—A human TOP3α cDNA was obtained by PCR using a human fetal brain cDNA library and primers, 5′-ATGATGCTTCTCTCTCGGCCC-3′ and 5′-TGTGTGGAGGGCAAGAGAAACAC-3′. The PCR product was used to make a digoxigenin-labeled probe by random priming (DIG DNA Labeling Kit, Boehringer Mannheim). The probe thus obtained was used to screen a mouse brain cDNA library (oligo(dT)-primed). Two positive clones were obtained from approximately 1 × 10⁶ clones, and Southern blot and sequencing analyses indicated that both of them encoded a protein that had homology with human TOP3α lacking the 5′-terminal region but not TOP3β itself. To obtain the 5′-terminal region, PCR on a T-cell line cDNA library was performed using primers, 5′-CTGTTAGAGCGTGGAGAATCAAATG-3′ and 5′-ATTGAGATGGTGCCACGATGC-3′ (the sequence derived from the vector), and gave a cDNA fragment (nucleotide numbers, 688–1477). Additional PCR from the mouse spermatocyte cDNA library was performed using primers, 5′-ACCCAGCTAGGTTCTGGTGTTAGG-3′ and 5′-TTGACACCCAGCAAATGTTAAG-3′ (the sequence derived from the vector), and a cDNA fragment (1–842) was obtained. These cDNA fragments were connected by PCR and subcloned in the pGEM-T Easy vector to generate mTOP3β/pGEM. Finally, the

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sequence of mTOP3β cDNA was confirmed by direct sequencing of the reverse transcription-PCR product from total RNA extracted from the testis of BALB/c mice.

Generation of a Recombinant Baculovirus Harboring mTOP3β cDNA—mTOP3β gene was amplified by PCR from mTOP3β/pGEM-T using primers, 5′-GAGAATTCATGAAGACCGTGCTCATGGTAG-3′ and M13 reverse primer and subcloned into a pFASTBAC-HTa transfer vector using the EcoRI site. A recombinant baculovirus DNA containing mTOP3β cDNA was constructed by a BAC-TO-BAC baculovirus expression system (Life Technologies, Inc.) in accordance with the instruction manual.

Purification of mTopo IIIβ—About 80% confluent Trichoplusia ni, High Five cells (Invitrogen) cultured in 100-mm dishes, were infected with the recombinant baculovirus and cultured for an additional 72 h. Cells were harvested by centrifugation, washed, and resuspended in hypotonic buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl$_2$, 0.5 mM CaCl$_2$, 1% Nonidet P-40) containing protease inhibitors (1 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine hydrochloride, 1 mM NaHSO$_3$). The cell suspension was incubated on ice for 10 min, and centrifuged at 1,000 × g for 10 min. The pellet was resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl$_2$) containing the protease inhibitors and kept standing on ice for 10 min. The suspension was incubated at 14,000 × g for 20 min, and the supernatant was loaded onto a pre-equilibrated immobilized metal affinity chromatography (IMAC) TALON column (CLON-TECH). The column was washed with washing buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol) containing the protease inhibitors, and the proteins bound to the column were eluted with an elution buffer (50 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 100 mM imidazole) containing the protease inhibitors. The eluate was dialyzed against a medium containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and stored at −80 °C.

Assay for DNA Topoisomerase Activity—DNA topoisomerase activity was assayed by measuring the negatively supercoiled DNA relaxing activity. The standard reaction mixture (15 μl) for the DNA relaxation assay consisted of 40 mM Tris-HCl, pH 7.5, 1 mM MgCl$_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA, and 0.2 μg of supercoiled pBR322 DNA. For the assay of the activity of Topo I, the reaction mixture consisted of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.1 mg/ml BSA, 0.2 μg of DNA, and 10 units of Topo I from HeLa cells. The incubation proceeded at 37 °C unless otherwise indicated, and the reaction was stopped with 2 μl of a stop solution (1% SDS, 50% glycerol, 0.05% bromphenol blue). The sample was then loaded onto a 0.8% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA). After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination.

Fig. 1. Alignments of amino acid sequences of mouse and human TOP3β, mouse TOP3α, and yeast TOP3. The shading indicates the conserved amino acid among the Topo III proteins, and the boxing indicates the putative active site of type I topoisomerase. Human Topo IIIβ is the putative gene product of "gene 2" appearing in the Ref. 13.
RESULTS AND DISCUSSION

cDNA Cloning of mTOP3β—In the course of cloning the mouse homologue to human TOP3α, we isolated cDNA fragments encoding a protein that had homology to human TOP3α but was distinct from mTOP3α. Thus, we cloned cDNA covering the full-length of the putative TOP3. The translation initiation site (AAGA ATG AAG) has a favorable context for efficient translation initiation, since there is an A residue at the -3 position and an A residue at the +4 position (16). The nucleotide sequence contains an open reading frame of 863 amino acids, and the deduced molecular mass of the coded protein is 96.9 kDa. The deduced amino acid sequence showed homology to human and mouse Topo IIIα and Saccharomyces cerevisiae Topo III. Recently, a human genomic sequence containing a putative ORF encoding a protein possessing a homology with Topo IIIα has been reported and this gene was named TOP3β (12, 13). Thus the deduced amino acid sequences were compared between the novel mouse TOP3 and the human TOP3β. The result of alignment of amino acids using the BLAST program indicated that the mouse novel TOP3 has an 86% amino acid identity with the human TOP3β (Fig. 1). Thus we named the novel mouse TOP3 as mTOP3β.

Purification of Recombinant mTopo IIIβ and Detection of DNA Topoisomerase Activity—To examine whether the mTOP3β gene product has DNA topoisomerase activity, mTopo IIIβ...
IIIβ was expressed in insect cells using a recombinant baculovirus DNA harboring mTOP3β cDNA with a hexahistidine tag and was purified with an IMAC column. mTopo IIIβ was eluted from the column at 100 mM imidazole by stepwise elution. An analysis by SDS-PAGE indicated that the purified fraction contained a single band of about 100 kDa (Fig. 2).

We assayed the DNA topoisomerase activity of mTopo IIIβ using the purified fraction. A partial relaxation of negatively supercoiled DNA by mTopo IIIβ was observed at 37 °C. Fig. 3A shows the time course of the reaction. A partial relaxation of supercoiled DNA was observed after incubating for only 1 min. Although the amount of partially relaxed DNA was increased during incubation in a time-dependent manner, the change in the linking number of partially relaxed DNA was very slow, and the amount of completely relaxed DNA was very small even after incubation for 15 min (Fig. 3A, left panel). These results indicate that the relaxation of DNA becomes harder as the number of supercoiling of DNA decreases, namely, the chance to expose a single-stranded portion decreases.

DNA relaxing activity of mTopo IIIβ was also confirmed by performing electrophoresis in a chloroquine-containing gel. As shown in the right panel of Fig. 3A, the amount of fast migrating DNA increased in a time-dependent manner.

We next assayed the enzyme activity at various temperatures from 37 to 62 °C (Fig. 3B). The change in linking number was observed at all the temperatures tested up to 62 °C. The number of DNA molecules that changed their linking number decreased upon rising incubation temperatures. However, complete relaxation of DNA became prominent at temperatures higher than 47 °C. These results seem to indicate that mTopo IIIβ becomes more unstable at higher temperatures, but once bound to DNA, it is stabilized and can continue relaxation of DNA, because the chance to expose a single-stranded portion increases at higher temperatures.

Fig. 3C shows the magnesium requirement for the topoisomerase activity. In the presence of 5 and 10 mM Mg2+ more DNA molecules were relaxed than in the presence of 1 mM Mg2+ or in its absence, but the levels of relaxation were lower under the former conditions, probably due to the difficulty in exposing a single-stranded portion. In the absence of Mg2+ and in the presence of 1 mM EDTA, complete relaxation was observed in a small portion of supercoiled DNA. The reason why relaxation occurs in the absence of Mg2+ is not clear at present. However, it is conceivable that a small portion of Topo IIIβ still contains divalent cations under the above conditions and performs relaxation under the conditions relatively easy to expose a single-strand portion.

To confirm the requirement of a single-stranded portion for mTopo IIIβ to exhibit activity, positively supercoiled DNA was used as the substrate. mTopo IIIβ was not able to relax positively supercoiled DNA which was relaxed by HeLa Topo I (Fig. 1D).

These results suggest that the preferable substrate for mTopo IIIβ, like yeast Topo III, might be a DNA containing a partially unwound region, which appears by the action of DNA helicases. In this respect, the report that yeast Topo III interacts with Sgs1 genetically and physically is interesting, because Sgs1 has DNA helicase activity (17).

Expression of mTOP3β mRNA—The expression of mTOP3β mRNA in various mouse tissues was examined by Northern blotting (Fig. 4A). An approximately 3-kilobase transcript was strongly expressed in the testis, relatively strongly in the brain, and weakly in other tissues including the heart, lung, thymus, and kidney. The mTOP3α mRNA was also expressed strongly in the testis, but in contrast to TOP3β, very weakly in other tissues as reported previously (15).

We next examined the expression of TOP3β mRNA in the testis using RNA extracted from the testes of mice of various ages because spermatogenesis occurs fairly synchronously in the testis after birth. It was found that the TOP3α mRNA, similar to the TOP3α mRNA, increased slightly 14 days and considerably 17 days after birth, when the cells in the pachytene phase begin to appear and increase (Fig. 4B). These results appear to indicate that Topo IIIβ is involved in some process of meiotic recombination probably with Topo IIIα, because meiotic recombination is known to occur in pachytene cells. Interestingly, this expression pattern is similar to that of mBLM (18). Thus it appears likely that these proteins function in the same developmental stages in the testis.

Recently, it has been reported that Topo IIIβ is essential in early embryogenesis in mice. It would be interesting to know whether Topo IIIβ is essential for the viability of cells like as Topo IIIα or not. We are now trying to make TOP3β-disrupted cells using a chicken B cell line, DT40 (19).

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