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# Characterization of cDNA encoding the mouse DNA topoisomerase II that can complement the budding yeast *top2* mutation

Noritaka Adachi[1,2], Mitsuko Miyaike[2], Hideo Ikeda[1] and Akihiko Kikuchi[2,*]
[1]Institute of Medical Science, University of Tokyo, PO Takanawa, Tokyo 108 and [2]Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan

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## ABSTRACT

**Several cDNA clones encoding mouse DNA topoisomerase II were obtained from a mouse spermatocyte cDNA library and the entire coding sequence of the gene was determined. The mouse DNA topoisomerase II consists of 1528 amino acids with a molecular weight of 173 kDa. It shares significant homologies with the other eucaryotic enzymes, although species-specific sequences are observed in their highly charged C-terminal regions. The complete mouse *TOP2* cDNA was put under yeast *GAL1* promoter and examined for complementation of *top2$^{ts}$* mutation in *S.cerevisiae*. We found that the cloned mouse gene could rescue the temperature-sensitive *top2$^{ts}$* mutation, depending on its induction by galactose. The functional expression of the mouse DNA topoisomerase II in yeast was further confirmed by enzymatic assays and by immunological methods with antibodies specific for the mouse enzyme.**

## INTRODUCTION

Eucaryotic DNA topoisomerase II (Topo II, also called type II DNA topoisomerase) can relax both positively and negatively supercoiled DNA and can also resolve catenated and knotted DNAs in the presence of ATP (1). The enzyme is of importance throughout the cell proliferation to provide the molecular tool for handling the constrained or entangled DNA which might be accumulated during DNA transactions such as replication, transcription or recombination (2–5).

In yeasts, in addition to biochemical studies, genetic approach has been quite successful to pin down the functional roles of DNA topoisomerases. DNA topoisomerase I, which can also relax supercoiled DNA in the absence of ATP, is not an essential enzyme (6–8). In contrast, DNA topoisomerase II is shown to be essential for the cell viability; it is required for chromosome condensation and segregation at mitosis (9–12) and for chromosome segregation at meiosis (13).

In higher eucaryotic cells, however, such an approach is not yet popular, because of difficulty in obtaining the deficient or the conditionally lethal mutant of DNA topoisomerases, especially in the case of DNA topoisomerase II. The mutant cell lines resistant to the drugs specific to DNA topoisomerase II were isolated in a few case (14), but they are still insufficient to characterize its *in vivo* functions. Since DNA topoisomerase II is an essential enzyme in yeast, the expression of a heterologous DNA topoisomerase II gene in yeast might be able to provide a system for analyzing the functions of the gene from other species. It is reported, indeed, that expression of *Drosophila* DNA topoisomerase II cDNA can rescue the conditionally lethal, temperature-sensitive mutations in the yeast DNA topoisomerase II gene (*TOP2*), as well as the mutations in which the *TOP2* locus was disrupted, although their amino acid sequences are only 46% homologous (15). So it seemed feasible to extend a similar method for the mammalian enzyme. Since the genetic devices in mammals are advanced in the mouse system, for example, gene targeting or transgenic mice, we aim to develop the system to collect as many mutations in the mouse *TOP2*. Here we show the complete cDNA sequence encoding the mouse DNA topoisomerase II and our results on the functional complementation of mutation in the yeast *TOP2* by the cloned mouse gene.

## RESULTS

### Isolation of cDNA clones encoding the mouse DNA topoisomerase II

It is well documented that the amount of DNA topoisomerase II is relatively high in efficiently proliferating cells (16, 17). In the usual growing cells, however, the enzyme turns over at extremely high rates, that is, the increment of the enzyme occurs only during $G_2$-M phase, followed by rapid decay prior to the next $G_1$ phase. This suggests that the mRNA might be unstable even in the rapidly growing cells.

During spermatogenesis the spermatocyte stays a long period at the $G_2$-M phase for the reductive cell division, suggesting that the amount of DNA topoisomerase II might be kept in a high level in the spermatocyte. Thus it is quite reasonable to expect the stable accumulation of the message in the spermatocyte which

* To whom correspondence should be addressed

would be a good source for the cDNA library in looking for the *TOP2* gene. This is why we surveyed the mouse spermatocyte cDNA library for the first choice.

Screening of the library in phage λgt11 was done by plaque hybridization with human *TOP2* cDNA as a probe. As expected, we could obtain many cDNA clones and complete cDNA sequence encoding the mouse DNA topoisomerase II was determined.

## Coding sequence of the mouse *TOP2* gene

The predicted mouse DNA topoisomerase II consists of 1528 amino acids and has a molecular weight of 173 kDa. When the amino acid sequence of the mouse enzyme deduced from the cDNA sequence is compared with that from human *TOP2* cDNA sequence (18), an extensive homology is found, that is, 89% of amino acid residues are identical between the mouse and human enzymes (Figure 1). It is apparent that the major difference resides only at the C-terminal 300 amino acids region, although both stretches show a similar hydrophilic nature. Furthermore, comparing the mouse enzyme sequence with those deduced from *TOP2* gene sequences of *Drosophila* (19), *S.cerevisiae* (20) and *S. pombe* (11, 21), the C-terminal one-third of the whole sequences of eucaryotic DNA topoisomerase II are divergent (Figure 2), even though overall homologies (55%, 46% and 46%, respectively) are significant.

It should be noted that the functionally important domains, suggested mainly by a comparison with DNA gyrase, a bacterial type II DNA topoisomerase (11, 19), are well conserved. As observed in other eucaryotic cells, mouse DNA topoisomerase II is also consisted of three regions: the N-terminal one-third homologous to the B subunit of DNA gyrase including a highly conserved ATPase domain (22−24), the central portion homologous to the A subunit of DNA gyrase containing a strictly conserved active site for breaking and rejoining (22, 25), and the highly charged C-terminal domain with clusters of acidic and basic residues. Furthermore, nuclear targeting sequences (26) and leucine zippers (27), pointed out by the sequence data from human DNA topoisomerase II (14, 28), are also found in the corresponding regions of the mouse enzyme (Figure 3).

## Expression of mouse *TOP2* cDNA in *E.coli*

To show the cDNA clones obtained here encode mouse DNA topoisomerase II, two clones mTOP2-AN and -BE, were put under T7 phage expression system (29) and the fragments were expressed in *E.coli*. In both cases a large quantity of protein corresponding to each fragment was obtained as one of the major protein constituents in *E.coli* crude lysates (Figure 4A(a)). By immunoblotting analysis, the protein product from the clone mTOP2-AN was strongly cross-reacted with antibody against human DNA topoisomerase II (Figure 4A(b) lane 1), indicating that the sequence is actually encoding DNA topoisomerase II. In contrast, the product from the mTOP2-BE was little detected by the same antibody (Figure 4A(b) lane 2). This fragment does not possess its major epitope region of the antibody against human DNA topoisomerase II (Miyaike *et al.*, unpublished results).

## Antibody against mouse DNA topoisomerase II

Since a large amount of protein was obtained from the small quantity of *E.coli* culture, we tried to raise the antibody against each protein product. The antisera were successfully obtained

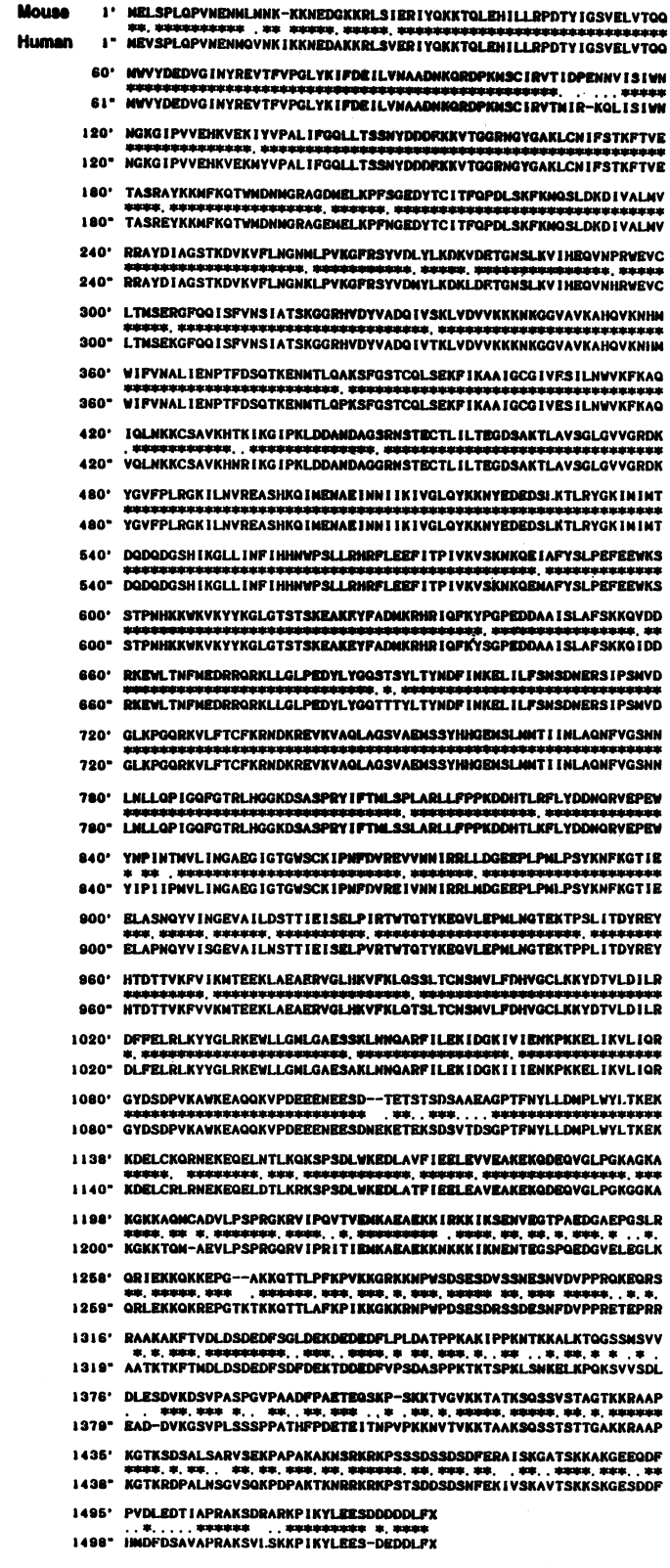


**Figure 1.** Amino acid sequence comparison between the mouse *TOP2* (upper) and the human *TOP2* (lower). Residue numbers are shown at the left side of the alignment. Identical residues are marked by asterisks (*) and conservative substitutions by dots(.). Gaps were introduced to maximize homology.

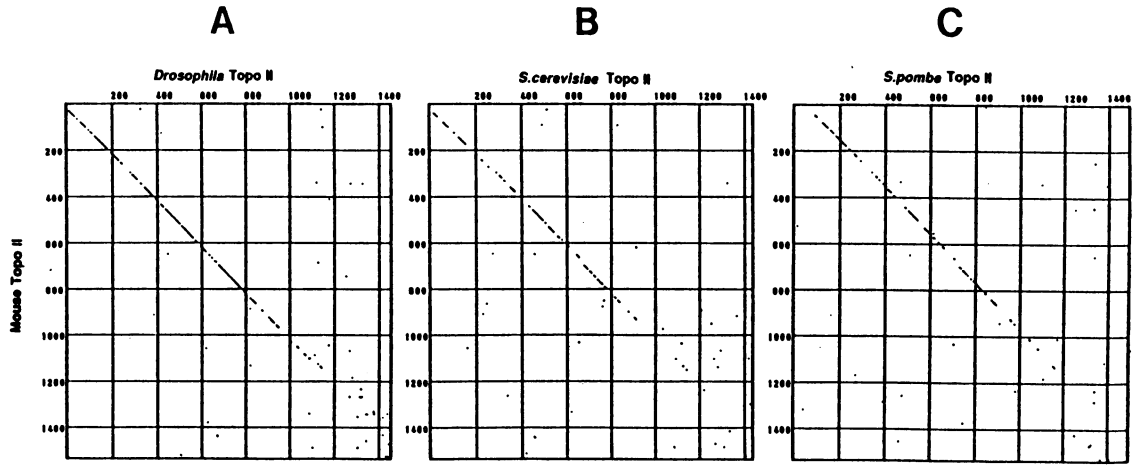


**Figure 2.** Amino acid sequence comparison of the mouse *TOP2* with *Drosophila TOP2* (A), *S.cerevisiae TOP2* (B) and *S. pombe TOP2* (C). The mouse *TOP2* sequence (1528 residues) is shown along the vertical axis from top to bottom. The other *TOP2* sequences (1447, 1427 and 1484 residues, respectively) are shown along the horizontal axis from left to right. Regions sharing homology (> 5 out of 7 residues are identical) produce a segmental dot.

in both cases, one (named #90) from the mTOP2-AN and the other (named #93) from the mTOP2-BE. Both antisera expectedly recognized each protein product expressed in *E.coli* (data not shown). Furthermore, by immunoblotting analysis, they specifically recognized a unique 170 kDa protein in the whole cellular extract of mouse L cell (Figure 4B lanes 1 and 3), while no protein band was detected in a human cellular extract (Figure 4B lanes 2 and 4). From these results, it is apparent that the cDNA clone we obtained here surely encodes the mouse DNA topoisomerase II, and the antisera thus obtained are specific to the mouse enzyme.

## Functional complementation of the yeast *top2* mutation by the mouse *TOP2* cDNA

First, the complete mouse *TOP2* cDNA that contains the entire coding sequence was reconstructed in pTV118N vector (Takara) from the four partial cDNA clones, mTOP2-N1, -N3, -BE and -C9, generating the plasmid pTVmTOP2. Using pTVmTOP2 as a template, the cloned mouse *TOP2* was amplified by polymerase chain reaction (PCR) to generate the suitable restriction sites at both ends and the PCR product was cleaved with *Hin*dIII and *Sph*I. So the 4.6 kb *Hin*dIII-*Sph*I fragment containing the mouse *TOP2* was recloned to a multi-copy shuttle vector pYES2 (Figure 5A). The plasmid pYESmTOP2 thus constructed was used for the expression of the mouse *TOP2* cDNA in *S.cerevisiae*. The expression of the gene is under the control of the yeast *GAL1* promoter: the transcription from this promoter should be induced by adding galactose in the medium and repressed by glucose (30).

The yeast strain SD1-4, containing a temperature-sensitive mutation in the *TOP2* gene (9), was transformed with either the expression plasmid pYESmTOP2 or the control vector pYES2, and plated at the permissive temperature (27°C). The resulting Ura$^+$ transformants were isolated and examined for complementation of the temperature-sensitive phenotype (Figure 5B). All the Ura$^+$ transformants grew at the permissive temperature on both glucose and galactose plates. At the restrictive temperature (37°C), however, the cells carrying the

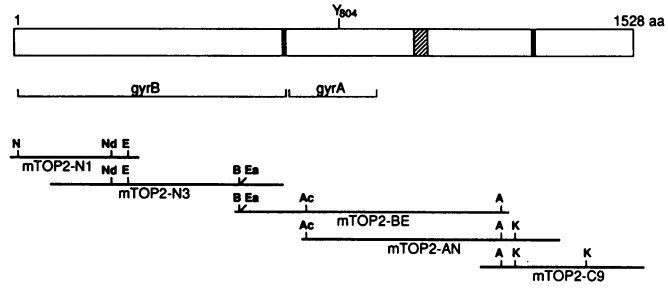


**Figure 3.** Schematic representation of mouse *TOP2* cDNA. The box shows the open reading frame. The black and hatched boxes represent the nuclear targeting sequences and leucine zipper motif, respectively. Position of the active site tyrosine is indicated. Regions homologous to the bacterial gyrase subunits A (*gyrA*) and B (*gyrB*) are shown. Five cDNA clones of the mouse *TOP2* are shown below. Selected restriction sites are indicated: A, *Aat*II; Ac, *Acc*I; B, *Bal*I; E, *Eco*RI; Ea, *Eae*I; K, *Kpn*I; N, *Nco*I; Nd, *Nde*I. All fragments are bounded by *Eco*RI sites generated during cDNA cloning. aa, amino acids.

control vector pYES2 failed to grow on either plate, while the cells transformed with the expression plasmid pYESmTOP2 did grow normally on galactose plate, where the expression of the mouse gene should be induced. Furthermore, at the restrictive temperature, this *top2$^{ts}$* strain carrying pYESmTOP2 could only grow in a galactose-containing medium and its growth was suppressed by glucose (data not shown). These results show that the yeast *top2$^{ts}$* mutation can be rescued by the functional expression of mouse *TOP2*.

## Expression of mouse DNA topoisomerase II activity in yeast

By immunoblotting analysis, we monitored the expression of the mouse gene in the *top2$^{ts}$* strain harboring the plasmid pYESmTOP2 (Figure 5C(a)). As expected, the cells accumulated the mouse DNA topoisomerase II during growth in a galactose-containing medium but not during growth in the glucose, judging from the detection with the antibody (# 90) against mouse DNA topoisomerase II. Although proteolysis seemed to occur in the

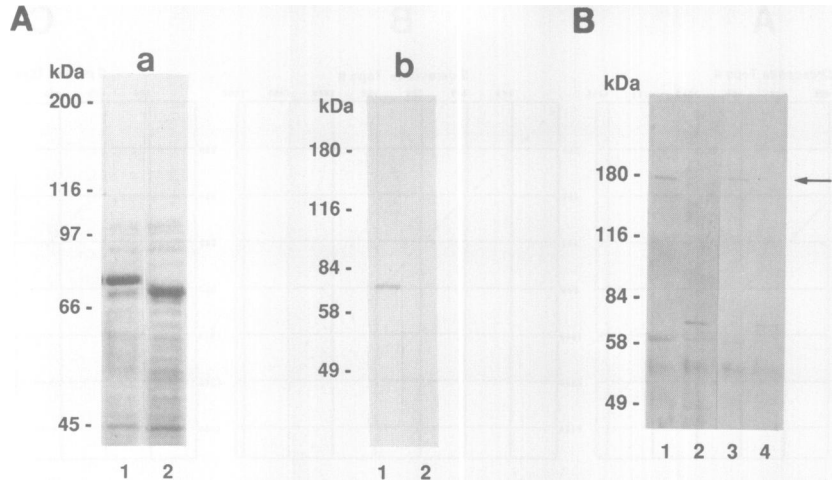


**Figure 4.** (A) Expression of mouse *TOP2* cDNA in *E.coli* by the use of T7 phage expression system. Crude lysates (1μl in panel a and 0.05 μl in panel b) of *E.coli* carrying the clone mTOP2-AN (lane 1) or mTOP2-BE (lane 2) were examined by staining with Coomassie Brilliant Blue (a) and by immunoblotting using antibody against human DNA topoisomerase II (b). Positions of molecular weight standards are shown on the left side. (B) The specificity of antibodies against the mouse DNA topoisomerase II. The cellular extracts (50 μg), prepared from mouse L cell (lanes 1 and 3) and human A431 cell (lanes 2 and 4), were analyzed by immunoblotting using polyclonal antibodies raised against the protein product of the clone mTOP2-AN (lanes 1 and 2) or mTOP2-BE (lanes 3 and 4). The arrow indicates the position of mouse DNA topoisomerase II. Positions of molecular weight standards are shown on the left.

yeast crude extracts, the full length product surely migrated at the expected position.

We also examined the activity of mouse DNA topoisomerase II in the extracts by decatenation assay (Figure 5C(b)). The yeast cells harboring pYESmTOP2 were grown, in the presence of either galactose or glucose, at the permissive temperature and then the activity was assayed at 37°C. At this temperature, the yeast DNA topoisomerase II activity is much reduced, since the temperature-sensitive mutation presents in the structural gene for yeast DNA topoisomerase II (9). As shown in Figure 5C(b), in the extract from the cells grown in a glucose-containing medium, the DNA topoisomerase II activity was not detected (lanes 1 and 2). In contrast, the cells grown in the galactose did exhibit high activity only in the presence of ATP (lane 4), and not in the absence of ATP (lane 3).

These data strongly suggest that the mouse *TOP2* gene is actually expressed as a functional form in the budding yeast and this activity rescues the yeast *top2$^{ts}$* mutation.

## DISCUSSION

We have obtained several cDNA clones containing the coding sequences of mouse DNA topoisomerase II from a mouse spermatocyte cDNA library by hybridization with human *TOP2* cDNA as a probe. From these clones the entire coding sequences of the mouse *TOP2* gene were determined.

The initiation codon of the mouse *TOP2* gene is tentatively located at the simillar site to the start site of the human *TOP2* gene (18) (data not shown). The nucleotide sequence GTCA-CCATGG, in which the underlined ATG is the putative start codon, matches very well with the consensus sequence for initiation of translation in vertebrates, GCC$^{A}/_{G}$CCATGG (31).

The functionally important domains, namely, the two regions homologous to the bacterial DNA gyrase B subunit and A subunit, are well conserved in the mouse enzyme as well, including ATPase domains and the catalytic site tyrosine at position 804. It is interesting that putative leucine zippers exist in the mouse and human enzyme, but not in *Drosophila* and yeast enzyme. This might suggest that the mode of homodimer formation and DNA-binding of mammalian DNA topoisomerase II is different from those of lower eucaryotic enzymes. As mentioned above, the C-terminal domain of eucaryotic DNA topoisomerase II is divergent and species-specific, although showing similar hydrophilic nature. In this highly charged domain, there are a lot of putative phosphorylation sites and indeed, in *S.cerevisiae*, some of these sites are shown to be phosphorylated by casein kinase II *in vivo* (32). Considering that phosphorylation has been shown to stimulate DNA topoisomerase II activity *in vitro* (33−36), the C-terminal domain may be involved in functional regulation *in vivo* in a species-specific manner. Recently, most of the C-terminal domain of the *S. pombe* DNA topoisomerase II is shown to be dispensable for *in vivo* activity (21). Such works using the mouse *TOP2* clone would be stimulating.

Recently it has been demonstrated that there are two different isoforms of DNA topoisomerase II in human (28): one is the α isotype (170 kDa-form) that has previously been reported, and the other is a newly identified β isotype (180 kDa-form), although only its part has been cloned to date. So it is probable that there are two *TOP2* genes in mouse as well. The *TOP2* cDNA clone we obtained here is apparently an α isotype. The mouse cDNA shares a higher homology with the human α isotype, as compared to the human β isotype at nucleotide level, although the difference is not so clear when they are compared at amino acid sequence level (data not shown).

We have demonstrated that the expression of the mouse gene in yeast can rescue the conditional lethal mutation in the yeast *TOP2* gene. However, such a complementation might be mediated by formation of a heterodimer of mouse and yeast enzyme to suppress the temperature-sensitive lesion of yeast enzymes. To rule out this possibility, we should examine whether the cloned mouse *TOP2* can rescue a null allele of the yeast *TOP2* gene. If such complementation actually takes place, we could

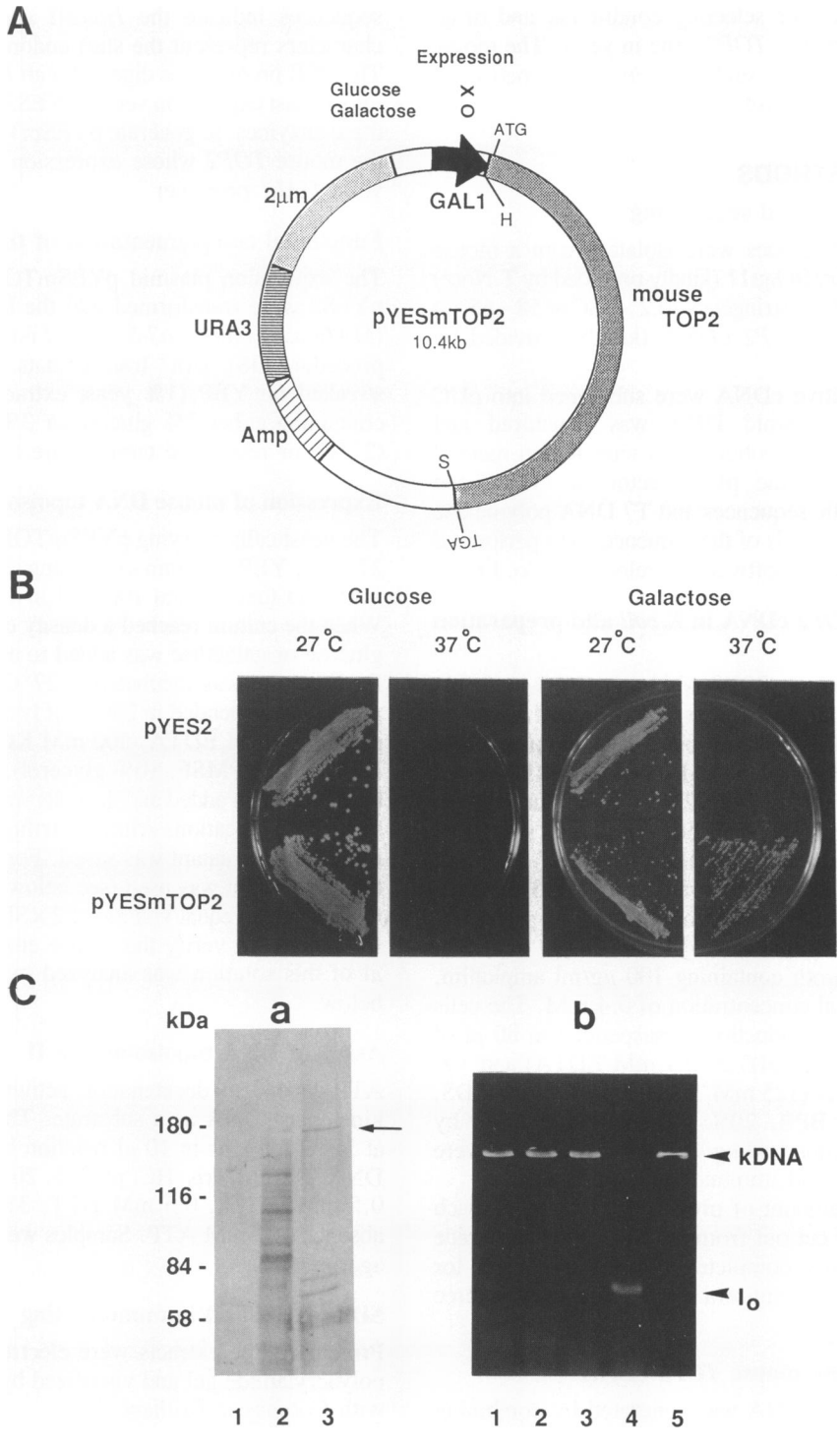


**Figure 5.** (A) Structure of the yeast expression plasmid pYESmTOP2. A 4.6 kb *Hin*dIII-*Sph*I fragment containing the mouse *TOP2* cDNA was ligated at the *Hin*dIII and *Sph*I sites of a shuttle vector pYES2 which contains the yeast *URA3* gene as a selectable marker. In this plasmid the mouse *TOP2* cDNA is expressed under the control of yeast *GAL1* promoter. Positions of initiation (ATG) and termination (TGA) codons are indicated. Amp, ampicillin-resistance gene; 2 μm, portion of yeast 2 μm plasmid; H, *Hin*dIII; S, *Sph*I; kb, kilobase. (B) Complementation of the yeast *top2* mutant by the mouse *TOP2* cDNA. Yeast strain SD1-4 was transformed with either pYESmTOP2 or pYES2. Transformants were streaked on YEP media containing glucose or galactose and incubated at 27°C or 37°C. The plates were photographed on the 2nd day (27°C) or on the 4th day (37°C) of incubation. (C) Expression of mouse *TOP2* gene in yeast. The yeast cells carrying pYESmTOP2 were grown in the presence of either glucose (lane 1 in panel a, lanes 1 and 2 in panel b) or galactose (lane 2 in panel a, lanes 3 and 4 in panel b). (a) Each of the cell lysates (15 μl) was analyzed by immunoblotting using polyclonal antibodies (#90) against mouse DNA topoisomerase II. Lane 3 contains the extracts from mouse L cell. The arrow indicates the position of mouse DNA topoisomerase II. Positions of molecular weight standards are shown on the left. (b) Detection of mouse DNA topoisomerase II activity in the extract. The reaction was carried out at 37°C in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of ATP. Lane 5, control (no extract added). The catenated network of kinetoplast DNA (kDNA) remains at the origin, while the monomer circles ($I_0$) decatenated by DNA topoisomerase II activity enter the gel.

develop a genetic system for selecting conditional and drug-resistant mutants of the mouse *TOP2* gene in yeast. The mouse clone will be very useful to study the *in vivo* function of mammalian DNA topoisomerase II.

## MATERIALS AND METHODS

### Isolation of cDNA clones and sequencing

The mouse *TOP2* cDNA clones were isolated from a mouse spermatocyte cDNA library in λgt11 (kindly provided by T.Noce) by screening at relatively high stringency (0.2×SSC at 58−65°C) with a full length human *TOP2* cDNA (kindly provided by J.C.Wang) as a probe.

The inserts in the positive cDNA were subcloned into pUC vectors (Takara). The plasmid DNA was denatured and sequenced by the dideoxynucleotide chain termination method (37) by using primers in the pUC vector or synthesized oligonucleotides at specific sequences and T7 DNA polymerase (Pharmacia). Computer analysis of the sequences was performed using GENETYX programs (Software Development Co. Ltd.).

### Expression of mouse *TOP2* cDNA in *E.coli* and preparation of antiserum

For expression of the clone mTOP2-AN in an IPTG-inducible bacterial vector, the 1.9 kb *Acc*I (blunt ended)-*Eco*RI fragment encoding amino acids 719−1355 of mouse DNA topoisomerase II was subcloned into *Nhe*I (blunt ended)-and *Eco*RI-digested pET-3c. For the clone mTOP2-BE, the 2.0 kb *Eae*I (blunt ended)-*Eco*RI fragment encoding amino acids 561−1229 was subcloned into *Bam*HI (blunt ended)-and *Eco*RI-digested pET-3c. Each plasmid was transformed into *E.coli* strain, BL21(DE3), and the mouse clone was expressed by induction through T7 promoter. Briefly, when the bacteria had grown to an $OD_{600}$ of 0.6 at 37°C in 1.5 ml of LB broth containing 100 μg/ml ampicillin, IPTG was added to a final concentration of 0.4 mM. The cells were pelleted 4 h after the induction, resuspended in 80 μl of TE buffer (10 mM Tris·HCl pH7.5, 0.1 mM EDTA) and 100 μl of 2×SDS sample buffer (125 mM TrisHCl pH 6.8, 2% SDS, 10 mM DTT, 0.004% BPB, 20% glycerol), and lysed by sonication for 5 min. After boiling for 3 min, aliquots were subjected to SDS-PAGE and immunoblotting analysis.

In both cases a large amount of protein was obtained. Each of the protein bands was cut out from the SDS polyacrylamide gel, mixed with Freund's complete adjuvant and used for immunizing rabbits. Positive antisera were obtained after three booster injections.

### Construction of complete mouse *TOP2* cDNA

A complete mouse *TOP2* cDNA was generated by combining overlapping region from four clones. Briefly the *Nco*I-*Nde*I fragment from mTOP2-N1, the *Nde*I-*Bal*I fragment from mTOP2-N3, the *Bal*I-*Aat*II fragment from mTOP2-BE and the *Aat*II-*Eco*RI fragment from mTOP2-C9 were joined together in pTV118N vector (Takara) by several subcloning steps. This plasmid was called pTVmTOP2. To construct the complete mouse *TOP2* with a *Hin*dIII site just upstream of the ATG codon and a *Sph*I site downstream of the stop codon, polymerase chain reaction (PCR) was carried out using oligonucleotides 5′-CGAAGCTTGGATCCATGGAGTTGTCACCGCTGCA-GCC-3′ and 5′-AACCCGGGCATGCCTCAGAAGAGGTC-GTCATCGTC-3′ on pTVmTOP2 template. The underlined sequences indicate the *Hin*dIII and *Sph*I sites and the bold characters represent the start codon of the mouse *TOP2* gene. The PCR product was digested with *Hin*dIII and *Sph*I, and ligated into a yeast expression vector pYES2 that had been digested with these enzymes, to generate pYESmTOP2. This plasmid contains the mouse *TOP2* whose expression is under the control of the yeast *GAL1* promoter.

### Functional complementation of the yeast *top2* mutation

The expression plasmid pYESmTOP2 and the control vector pYES2 were transformed into the haploid yeast strain, SD1-4 (*MATa ade1 ade2 ura3-52 top2-1* ts) (9) using the lithium acetate procedure (38). $Ura^+$ transformats were obtained at 27°C and streaked on YEP (1% yeast extract and 2% peptone) media containing either 2% glucose or 2% galactose at a permissive (27°C) or restrictive temperature (37°C).

### Expression of mouse DNA topoisomerase II activity in yeast

The yeast cells carrying pYESmTOP2 were grown overnight at 27°C in YEP medium containing 2% raffinose and 2% lactic acid, and then diluted 100-fold in 10 ml of the same medium. When the culture reached a density of $2-3\times10^7$ cells/ml, either glucose or galactose was added to a final concentration of 2%. Each culture was incubated at 27°C for further 12 h. The cell pellet was suspended in 200 μl of lysis buffer (20 mM, Tris·HCl pH 7.5, 1 mM EDTA, 500 mM KCl, 10 mM $NaHSO_3$, 1mM DTT, 1 mM PMSF, 10% glycerol). One-third volume of glass beads was then added and the cells were lysed by vigorous mixing and brief sonication. After centrifugation at 12000 rpm for 10 min, the supernatant was saved. For enzymatic assays 0.5 μl of the supernatant was used (see below). A part of the supernatant mixed with an equal volume of 2×SDS sample buffer was boiled for 3 min. To verify the mouse enzyme synthesis in yeast, 15 μl of this solution was analyzed by immunoblotting as shown below.

### Assay of DNA topoisomerase II

ATP-dependent decatenation activity was measured by using kinetoplast DNA as a substrate. The reaction was carried out at 37°C for 1 hr in 10 μl reaction buffer (75 ng of kinetoplast DNA, 50 mM Tris·HCl pH 7.5, 20 mM NaCl, 20 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 3% BSA) in the presence or absence of 1 mM ATP. Samples were electrophoresed in 0.9% agarose gels.

### SDS-PAGE and immunoblotting

Proteins in the extracts were electroporesed in a SDS /7.5 % polyacrylamide gel and visualized by staining the protein bands with Coomassie Brilliant Blue.

For immunoblotting analysis, proteins were transferred to a nitrocellulose filter, reacted with rabbit antiserum at a 1 : 100 dilution, incubated with peroxidase-conjugated secondary antibody, and detected by chloronaphthol (Konica Immunostain HRP Kit).

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