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Mutant isolation of mouse DNA topoisomerase IIα in yeast

Noritaka Adachi[1,2,+], 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

For characterizing *in vivo* functions of a mammalian protein, it is informative to obtain conditional mutations and apply them to the mouse genetic system. However, the isolation of conditional mutations has been quite difficult in cultured cells. We report here that functional expression of a heterologous mammalian gene in the yeast *Saccharomyces cerevisiae* provides a system for isolating mutated genes. We found that the cloned mouse *TOP2α* cDNA, which encodes mouse DNA topoisomerase II (topo II) α, could rescue the lethal phenotype caused by yeast *top2* null mutation. In order to generate and select temperature-sensitive mouse topo IIα, an expression plasmid was mutagenized *in vitro* and was transformed, using the plasmid shuffling method, into the yeast strain, in which the endogenous *TOP2* gene had been disrupted. We observed that one of such clone of yeast cells harboring a mutagenized mouse *TOP2α* showed temperature-sensitive growth. Enzymatic assays and sequencing analysis revealed that this phenotype was caused by the thermosensitive nature of the mutant mouse protein, which has isoleucine at amino acid 961 instead of threonine. Therefore we have isolated the first conditional mutation in the mouse *TOP2α*.

INTRODUCTION

Eukaryotic DNA topoisomerase II (topo II) is a ubiquitous nuclear enzyme that alters the topological structure of DNA and chromosomes by transiently breaking both strands of a DNA double helix, passing another segment of DNA duplex through the break and religating the two strands (1). The enzyme can relax both positively and negatively supercoiled DNA and can also catalyze the knotting/unknotting and the catenation/decatenation of closed DNA circles (1). Accordingly, the enzyme is of importance throughout cell proliferation providing the molecular tool for handling the constrained or entangled DNA which accumulate during DNA transactions such as replication, transcription or recombination (2–5). In addition to catalytic function, topo II has been implicated as a structural component of the interphase nucleus, probably linking chromatin to the nuclear matrix, and of the metaphase chromosome scaffold (6–8).

In yeasts, in addition to biochemical studies, genetic approaches using mutants have been quite successful in pinning down the functional roles of the topo II enzyme. *TOP2*, the structural gene for topo II, has been shown to be essential for cell viability (9,10). Specifically, in *top2* mutant cells, the cell cycle-dependent phenotype, that is, the blocking of nuclear division, is observed at the time of mitosis (11–14); chromosomes in the *top2* cells are pulled by the spindle but fail to separate. So the topo II activity is required at the time of cell division, most likely for chromosome segregation. In fission yeast, it is also required for chromosome condensation (14).

In mammalian cells, however, *in vivo* function of topo II remains obscure because of difficulty in obtaining mutant enzymes. Furthermore, it has been demonstrated that there are two different isoforms of mammalian topo II (15,16); one is the α isoform (topo IIα) that has previously been reported, and the other is the newly identified β isoform (topo IIβ). Although the two isoforms are structurally similar (72% identity in human) (17–19), they are genetically and biochemically distinct, and it is still unclear how they share the roles in the cellular events that require topo II activity.

What are the *in vivo* roles of mammalian topo II? Do the two isoforms have distinct cellular function? To address these questions, genetic analysis using mutants, as has been quite successful in yeast, would be very useful. Since yeast topo II is an essential enzyme, we expected that the functional expression of a heterologous *TOP2* gene in yeast might be able to provide a system for isolating mutants, as well as analyzing the functions, of the gene product if it could rescue the lethal phenotype caused by inactivation of yeast topo II. As the genetic devices in mammals are advanced in the mouse system, we aim to develop a system to collect mutations in the mouse gene encoding topo II. Here we show the functional complementation of yeast *top2* null mutation by the cloned mouse *TOP2α* cDNA. Furthermore, taking advantage of this heterologous complementation system, we have succeeded in isolating a conditional (temperature-sensitive) mutation in the mouse *TOP2α*.

*To whom correspondence should be addressed

+Present address: Kihara Institute for Biological Research, Yokohama City University, Nakamura-cho 2-120-3, Minami-ku, Yokohama 232, Japan

MATERIALS AND METHODS

Yeast manipulations

Yeast was grown in YPD (1% yeast extract, 2% peptone and 2% glucose), SD (0.67% Yeast Nitrogen Base without amino acids and 2% glucose) supplemented with appropriate amino acids and 0.004% adenine, or YEPR media (1% yeast extract, 2% peptone and 2% raffinose) supplemented when appropriate with either 2% glucose or 2% galactose. For solid media, 2% Bacto-agar was added. 5-FOA media for selection of yeast *ura3* cells were made as described (24). As to the galactose-containing plate, galactose and raffinose were added instead of glucose. Yeast transformation was performed using standard methods (27).

Construction of the yeast strain NAY113

A 5.5 kb *Bam*HI−*Spe*I fragment of pSCTOP2-1 (28) containing the yeast *TOP2* gene was ligated at the *Bam*HI and *Xba*I sites of a single-copy vector YCUp4 (29) which contains the *URA3* gene as a selectable marker, to generate YCp-ScTOP2. The plasmid YCp-ScTOP2 was transformed into the haploid yeast strain NNY11 (*MAT*a *lys2-801 ura3-52 trp1 leu2 his3*) (30). The resulting Ura$^+$ colonies, termed NAY101, were isolated and used to disrupt the chromosomal *TOP2* gene. A 1.1 kb *Bgl*II−*Nhe*I fragment of YRp7 (31) containing the *TRP1* gene was subcloned into *Bgl*II- and *Avr*II-digested pSCTOP2-1, to replace the central 2.7 kb region of the *TOP2* gene by the *TRP1* gene. The resulting plasmid was then digested with *Pvu*II, and the 2.3 kb fragment containing the interrupted *top2* gene was used to transform the strain NAY101 in order to disrupt its chromosomal *TOP2* locus. The Trp$^+$ transformants were isolated and examined by polymerase chain reaction (PCR; 32) to determine whether the interrupted *top2* gene was integrated to the chromosome by homologous recombination. The expected strain thus made was called NAY113.

Complementation of yeast *top2* null mutation by mouse *TOP2α*

To make the expression plasmid pNA811 for mouse topo IIα, a 5.4 kb *Spe*I−*Mlu*I fragment of pYESmTOP2 (20) containing the mouse *TOP2α* cDNA under the yeast *GAL1* promoter was ligated at the *Spe*I and *Dsa*I sites of a shuttle vector pRS415 (21), which contains the yeast *LEU2* gene as a selectable marker. Then the plasmid pNA811 or the control vector pRS415 was transformed into the strain NAY113. The Leu$^+$ transformants were isolated and plated on 5-FOA media to examine whether they could form *ura3* colonies. Formation of *ura3* cells means that the mouse gene on the plasmid functionally substitutes for the yeast *TOP2*.

Isolation and characterization of the temperature-sensitive allele of mouse topo IIα

In vitro mutagenesis of the plasmid pNA811 was performed using hydroxylamine as described (23). Mutagenized plasmids were transformed into the strain NAY113 and 100 Leu$^+$ transformants were isolated and plated on galactose-containing 5-FOA media in order to remove the yeast *TOP2*. Each of the resulting *ura3* colonies (about 70) was then streaked on YEPR−galactose media and incubated at 25°C or 35°C. Only one strain, termed NAY159, failed to grow at 35°C, showing the temperature-sensitive growth.

Preparation of crude extracts were performed as follows. Yeast cells were grown overnight at 25°C in YEPR−galactose medium to a density of 5×10^7 cells/ml. The cell pellet was suspended in 50 μl of lysis buffer (20). One-third volume of glass beads was then added and the cells were lysed by vigorous mixing and brief sonication. After centrifugation at 12 000 r.p.m. for 5 min, the supernatant was saved as the yeast crude extract. For enzymatic assays, 0.5 μl of the crude extract was used (see below). To verify the synthesis of mouse enzyme in yeast, 10 μl of the crude extract was mixed with an equal volume of 2×SDS sample buffer (20) and was boiled for 3 min, followed by immunoblotting analysis. Proteins in the extracts were electrophoresed in a SDS/10% polyacrylamide gel and were transferred to a nitrocellulose filter, reacted with the monoclonal antibody 7B9 against the mouse topo IIα (N.Nozaki *et al.*, unpublished material. Note that this antibody recognizes the N-terminal regions of mammalian topo II protein and does not cross-react with the yeast enzyme) at a 1:10 dilution, incubated with peroxidase-conjugated secondary antibody, and detected by chloronaphthol (Konica Immunostain HRP Kit).

Identification of the mutation conferring the temperature sensitivity was carried out as follows. The strain NAY159 was grown in YEPR−galactose medium and the mutagenized plasmid was recovered (33). Since a chimeric gene analysis revealed that the mutation should be located within the central 2.9 kb *Nde*I−*Aat*II region of the mouse *TOP2α* (data not shown), appropriate restriction fragments of this region were subcloned into pUC vectors, followed by determination of the position of the mutation by dideoxy sequencing (34). It was found that a mutation of C T at nucleotide 2882 was the only alteration observed in the mutant gene compared with the wild-type gene. We have to refer to the findings that the mouse *TOP2α* cDNA nucleotide sequence reported previously (20) (D12513) missed a C (after nucleotide 2534) and additionally contained an A (at nucleotide 2531), and that nucleotide 2522 is a T rather than A. As to the nucleotide numbers, the A in the initiation methionine codon was numbered as 1.

Assay of topo II

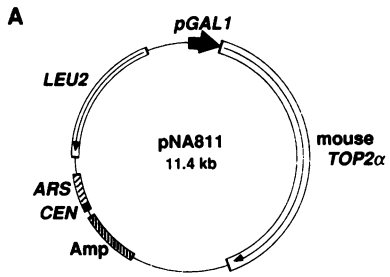
ATP-dependent decatenation activity of topo II was measured by using kinetoplast DNA as a substrate. The reaction was carried out at 35°C for 1 h in 10 μl reaction buffer (100 ng of kinetoplast DNA, 50 mM Tris−HCl pH 7.5, 20 mM NaCl, 20 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.003% BSA, 1 mM ATP). Samples were electrophoresed in 1.0% agarose gels.

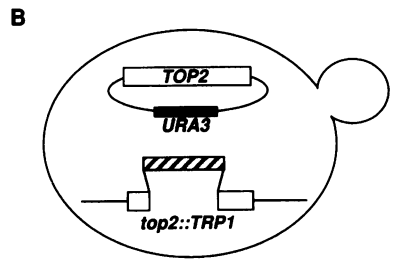
RESULTS

Functional complementation of yeast *top2* null mutation by mouse *TOP2α*

We reported recently the isolation of the full-length cDNA encoding the mouse topo IIα and showed that the functional expression of the cloned mouse gene (*TOP2α*) could rescue the temperature-sensitive *top2* mutation in yeast (20). Such complementation, however, 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 examined whether the cloned mouse *TOP2α* could rescue the yeast *top2* null mutation, where the yeast *TOP2* gene had been largely deleted.

The expression plasmid that produces the mouse topo IIα was constructed by putting the mouse *TOP2α* cDNA under the yeast *GAL1* promoter in a single-copy, *LEU2*-based vector pRS415 (21), designated pNA811 (Figure 1A). In this plasmid expression





C

transformed with	5-FOA plate	
	+glucose	+galactose
no plasmid	-	-
pRS415	-	-
pNA811	-	+

Figure 1. Complementation of the yeast *top2* null mutation by mouse *TOP2α*. (**A**) Structure of the yeast expression plasmid pNA811. A 5.4 kb *SpeI*−*MluI* fragment of pYESmTOP2 (20) containing the mouse *TOP2α* cDNA under the yeast *GAL1* promoter was ligated at the *SpeI* and *DsaI* sites of a shuttle vector pRS415 (21), which contains the yeast *LEU2* gene as a selectable marker. In this plasmid the mouse *TOP2α* is expressed under the control of yeast *GAL1* promoter. Amp, ampicillin-resistance gene; *CEN*, centromere; *ARS*, autonomously replicating sequence. (**B**) Schematic representation of the yeast strain NAY113. A yeast strain was transformed with a single-copy, *URA3*-based plasmid bearing the yeast *TOP2* gene and then most part of its chromosomal *TOP2* locus was deleted and replaced by the yeast *TRP1* gene (represented by *top2::TRP1*). (**C**) Viability of NAY113 on 5-FOA media. The strain NAY113 was transformed with either the expression plasmid pNA811 or the control vector pRS415, and the resulting Leu$^+$ cells were isolated and plated on 5-FOA media containing glucose or galactose. +, formed colonies; −, failed to form colonies.

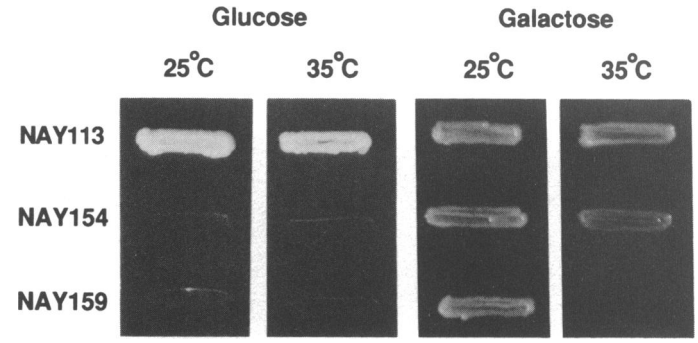


Figure 2. Temperature-sensitive growth of the strain NAY159. Yeast strains were streaked on media containing either glucose or galactose and incubated at 25°C or 35°C. The plates were photographed on the fifth day of incubation.

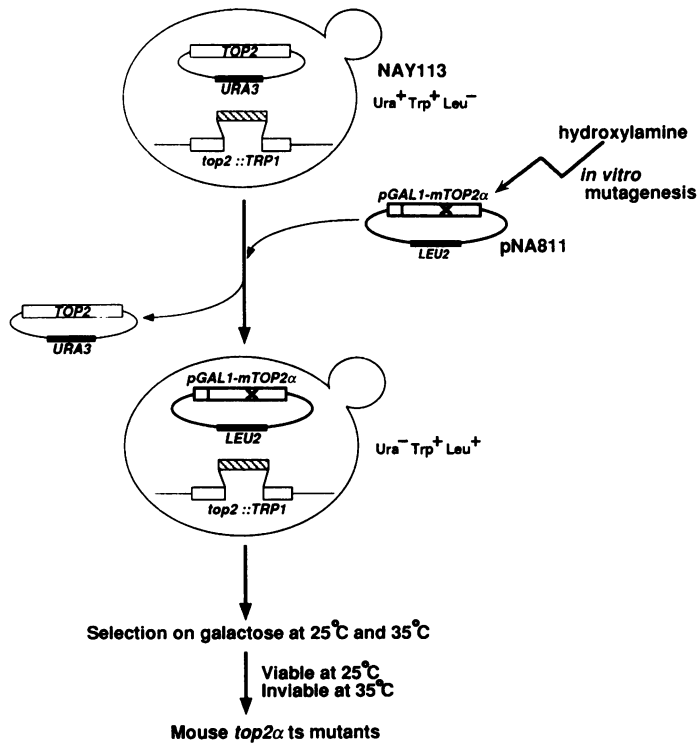


Figure 3. Strategy for isolating temperature-sensitive allele of mouse topo IIα. The plasmid shuffling technique, commonly used in yeast to isolate conditional mutations was applied to the heterologous mouse gene. The expression plasmid pNA811 was mutagenized *in vitro* by hydroxylamine and transformed into the yeast strain NAY113. The Leu$^+$ transformants were isolated and each of them was plated on galactose-containing 5-FOA plate in order to select the *ura3* cells. Some of the viable Ura$^-$ Leu$^+$ colonies was then streaked on galactose media and incubated at 25°C or 35°C to examine whether it would show a temperature-sensitive phenotype. Since the yeast topo II had been completely inactive, the conditional growth should be due to a mutation in the mouse gene. *pGAL1*−*mTOP2α* indicates the mouse *TOP2α* cDNA under the control of yeast *GAL1* promoter. The other symbols are as in Figure 1B.

of mouse *TOP2α* is induced by galactose and repressed by glucose (22). We employed the plasmid shuffling method (23) to replace the yeast *TOP2* by the mouse gene. First, we prepared the yeast strain, NAY113, in which the chromosomal *TOP2* locus was completely disrupted, but the cell was saved by a single-copy, *URA3*-based plasmid bearing the yeast *TOP2* gene (Figure 1B). This strain grew normally since the plasmid-born yeast topo II was active (Figure 2). On media containing 5-fluoroorotic acid (5-FOA) which selectively kills the *URA3* cells (24), it failed to grow (Figure 1C, no plasmid), because 5-FOA-resistant cells had lost the plasmid bearing the *TOP2* gene, being *top2* null mutants. It is noticed that we obtained no *ura3 TOP2*$^+$ cells in this system, indicating that no mutation event had been occurred in the *URA3* gene itself. Then the strain NAY113 was transformed with either the expression plasmid pNA811 or the control vector pRS415, and the resulting Leu$^+$ cells were isolated and plated on 5-FOA media supplemented with glucose or galactose. As shown in Figure 1C, the cells transformed with pRS415 failed to grow on either plate. The cells with pNA811, however, formed 5-FOA-resistant (*ura3*) colonies only on galactose-containing 5-FOA plate where the mouse *TOP2α* should be expressed. Furthermore, the *ura3* cells, termed NAY154, which grew normally on galactose plate, could not grow on the glucose (Figure 2). Thus the growth of NAY154 was galactose-dependent, suggesting that yeast *TOP2* was not remaining in this strain, as was confirmed by PCR analyses (data not shown).

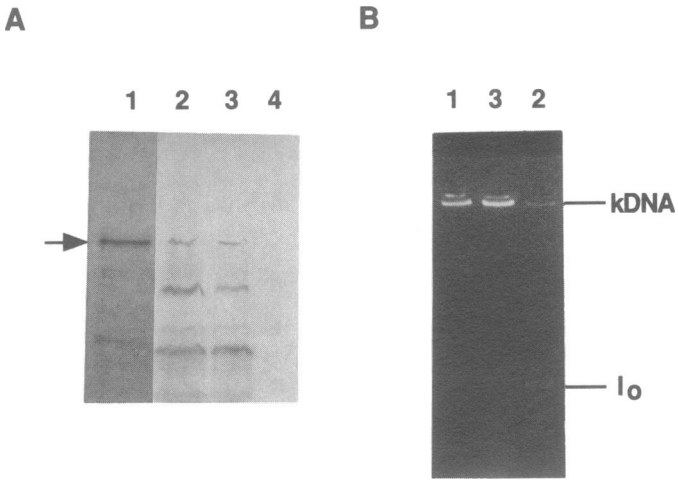


Figure 4. Characterization of mouse topo IIα in yeast crude extracts. Yeast cells were grown in YEPR−galactose medium and the crude extracts were prepared as indicated in Materials and Methods. (**A**) Each of the cell extracts was analyzed by immunoblotting using the antibody against mouse topo IIα. Lane 1 contains the extract (50 μg) from mouse L cells. Lanes 2, 3 and 4 contain the extract from NAY154, NAY159 and NAY113, respectively. The position of the protein is shown on the left. (**B**) Detection of topo II activity in the extract. The assay was carried out at 35°C. Lane 1, control (no extract added); lane 2, NAY154; lane 3, NAY159. The catenated network of kinetoplast DNA (kDNA) remains at the origin; while the monomer circles (Io) decatenated by topo II activity enter the gel, as is seen only in lane 2.

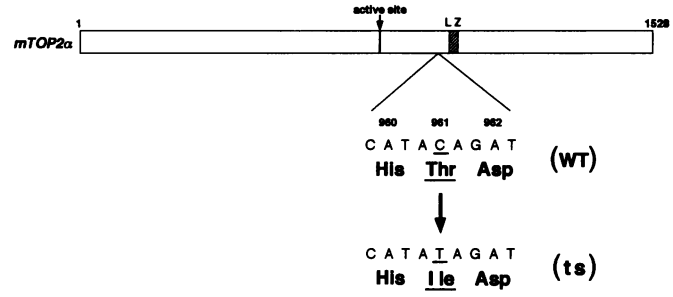


Figure 5. Characterization of the temperature-sensitive mouse *TOP2α*. The mutation locates immediately upstream of the leucine zipper motif. The C 2882 to T transition, which would change the threonine 961 to isoleucine, renders mouse topo IIα temperature sensitive. The hatched box represents the leucine zipper motif. Position of the active site tyrosine is indicated. The figures represent the residue numbers.

These results show that the mouse topo IIα rescues the lethal phenotype caused by the yeast *top2* null mutation and that yeast topo II can be functionally substituted by mouse topo IIα.

Isolation of temperature-sensitive mutant allele of mouse topo IIα

The plasmid shuffling technique has been used to isolate conditional mutations in the essential gene of interest (23). Since the mouse *TOP2α* replaces the yeast *TOP2*, this complementation system should have provided a simple system for generating and selecting conditional mutations in the mouse gene. We extended, therefore, the technique for the heterologous mammalian gene.

The method is summarized in Figure 3. The expression plasmid pNA811 was mutagenized *in vitro* using hydroxylamine and trasformed into the yeast strain NAY113. The Leu^+ transformants were isolated and each of them plated on galactose-containing 5-FOA plate in order to select the *ura3* cells. In this case, of course, not all the Leu^+ cells formed colonies since some had incurred mutations in the mouse *TOP2α*. Each of the resulting Ura^- Leu^+ colonies was then streaked on galactose media and incubated at 25 or 35°C to examine whether it shows a temperature-sensitive phenotype. As expected, a strain, termed NAY159, which grew normally at 25°C, failed to grow at 35°C, representing the temperature-sensitive growth (Figure 2). Considering that yeast topo II was completely inactive, this conditional lethality should be due to a mutation in the mouse gene. To confirm this, the yeast crude extracts were prepared from the two strains, NAY154 and NAY159, and used for characterization of the mouse gene products (Figure 4). We monitored the expression of the mouse gene in those strains by immunoblotting analysis using the antibody against the mouse enzyme. As shown in Figure 4A, both cells accumulated the mouse enzyme at the similar level (lanes 2 and 3). We then examined the activity of mouse topo IIα by decatenation assay at 35°C (Figure 4B). In the strain NAY154, which possessed the wild-type mouse enzyme, topo II activity was detected clearly in terms of decatenated monomer circles (Io) (lane 2). In contrast, in the strain NAY159 which showed the temperature-sensitive growth, the activity was not detected (lane 3). These data suggest that the mouse *TOP2α* in NAY159 has a mutation which causes its product to be temperature-sensitive. Sequence analysis has shown that this mutation is a C to T transition at nucleotide 2882, which would change threonine to isoleucine at amino acid 961 (Figure 5).

Thus we have established a yeast expression system for selecting conditional mutations in the heterologous mammalian gene. And using this system, we have succeeded in isolating the mutant mouse *TOP2α* whose product shows the temperature sensitivity.

DISCUSSION

Many efforts have been paid to isolate mutations in mammalian gene in cultured cells, but almost in vain except when the gene of interest is linked to X chromosome. In yeast, the plasmid shuffling technique, combined with *in vitro* mutagenesis, has been useful in isolating conditional mutations in genes of interest. We have extended this technique to the mouse *TOP2α* gene which can be functionally expressed in yeast cells, and have isolated, for the first time, a conditional mutation in the mouse *TOP2α* whose product shows the temperature sensitivity *in vitro*, as well as *in vivo*. It should be noted that we could not detect topo II activity at 25°C *in vitro* even in the wild-type strain, since the assay did not work at this temperature.

The mutant allele obtained here had a point mutation at 2882 nt (C→T), which would change the threonine 961 to isoleucine. Since this mutation is located immediately upstream of the leucine zippers (20,25), one could speculate that disruption of this structure may demolish protein folding or subunit interaction. It is interesting to note that the threonine 961 residue is conserved in the mouse topo IIβ as well (N.Adachi *et al.*, in preparation), so that its enzymatic activity might be temperature-sensitive if the same mutation were introduced.

In the yeast *Saccharomyces cerevisiae*, a number of temperature-sensitive *top2* mutants have been isolated and characterized so far. Thomas *et al.* (26) have found that the mutations conferring the thermosensitivity are usually single missense mutations, all of which appear to be located in the conserved region of topo II protein that shows homology to the A subunit of bacterial DNA gyrase. Two mutations, leucine 708 to proline and proline 821 to glutamine, are located in the amino acid residues that are conserved in all the type II DNA topoisomerases cloned to date. We expected that if either of the same mutations (leucine 729 to proline or proline 842 to glutamine) were introduced into the mouse *TOP2α*, then the protein product might be temperature-sensitive. We, therefore, performed this experiment by site-directed mutagenesis. However, our attempt was not successful, since the resulting mouse proteins could not rescue the yeast *top2* null mutation and had no enzymatic activity at any temperature (data not shown). Therefore we undertook the isolation of conditional mutations in the mouse gene by the method of *in vitro* chemical mutagenesis.

We have established a simple system for generating and isolating mutants of the mouse topo IIα in yeast. Since the enzyme might be essential for viability and any deletion or knockout mutations might not be obtainable, the conditional mutations would be greatly useful to analyze the *in vivo* roles of topo II if they could be applied to the mouse genetic system (for example, gene targeting or transgenic mice). Specifically, if mutant ES cells, in which both chromosomal *TOP2α* loci had been disrupted, could be obtained harboring the conditional *top2α* mutation, then such cell lines would be useful to pin down the *in vivo* roles of the enzyme. As we have already isolated a part of the genomic DNA containing the mouse *TOP2α* and have constructed a targeting vector for disruption of the *TOP2α* locus (N.Adachi *et al.*, unpublished results), such experiments will be performed soon. It will also be of interest to distinguish the functions of two isoforms of topo II, α and β.

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