Assignment of functional amino acids around the active site of human DNA topoisomerase IIα

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Running title, catalytic plane of human TOP2

Keywords, DNA gyrase / DNA topoisomerase / drug resistance / etoposide / random mutagenesis

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Abstract

An expression library for active site mutants of human topoisomerase IIα (TOP2α) was constructed by replacing the sequence encoding residues 793-808 with a randomized oligonucleotide cassette. This plasmid library was transformed into a temperature-sensitive yeast strain (top2-1) and viable transformants were selected at the restrictive temperature. Among the active TOP2α mutants, no substitution was allowed at Tyr805, the 5' anchor of the cleaved DNA, and only conservative substitutions were allowed at Leu794, Asp797, Ala801 and Arg804. Thus, these 5 residues are critical for human TOP2α activity, and the remaining mutagenized residues are less critical for function. Using the X-ray crystal structure of yeast TOP2 as a structural model, it can be deduced that these 5 functionally important residues lie in a plane. One of the possible functions of this plane may be that it interacts with the DNA substrate upon catalysis. The side chains of Ser803 and Lys798, which confer drug resistance, lie adjacent to this plane.
**Introduction**

Type II DNA topoisomerases are essential cellular enzymes that are required for cell proliferation. They catalyze topological change of DNA molecules through the transient breakage and rejoining of double-stranded DNA; mechanistically, the enzyme makes a gate in one double-strand DNA segment and allows another DNA segment to pass through the gate. These reactions relax supercoiled DNA and catenate or decatenate covalently-closed circular DNA molecules (1). In addition, type II DNA topoisomerases are clinical targets for antibiotics and anticancer drugs.

Prokaryotic DNA TOP2, also known as DNA gyrase, is composed of two subunits, A protein (GyrA) and B protein (GyrB), and the active form is an A$_2$B$_2$ heterodimer. Eukaryotic TOP2 is a homodimer composed of a monomer with two domains, B' and A', corresponding to DNA gyrase B and A subunits, respectively (2). All TOP2 enzymes share sequence similarity (3); in addition, similarity has been observed in the X-ray crystal structures of TOP2 proteins (4, 5). Because TOP2 is conserved through evolution, limited genetic interchangeability for TOP2 from different species is observed (6, 7, 8).

The enzymatic mechanism of TOP2 has been studied extensively, and it is thought to involve multistep conformation changes of the enzyme (9). Because only a small number of crystal structures of TOP2 have been published, it is difficult to understand the enzymatic mechanism fully from this structural information alone. However, biochemical experiments have been carried out to provide information on the interactions between the enzyme and its DNA substrate. For example, Worland and Wang showed that the active site Tyr783 attaches covalently to the 5' end of the cleaved DNA chain (10). Recent work on DNA gyrase suggested specific interactions between the enzyme and its DNA substrate at distinct steps of the catalytic cycle by probing the topology of the enzyme-bound DNA segment (11).
To understand enzymes and their biological function, it is important to characterize both their catalytic and their structural properties. Several experimental approaches are commonly used for this purpose. One approach is to determine the primary structures (i.e., protein sequences) of related proteins from different species and align them with one another. Such a sequence alignment is useful to identify the evolutionarily conserved amino acids and motifs that may play functional roles in a group of related proteins. X-ray crystallography and nuclear magnetic resonance are used to study the 3-dimensional structure of a protein at the atomic level. These structural methods provide information on the structure of the entire protein and its subdomains and subunits. Such information is necessary to understand how enzymes interact with substrates, other molecules, inhibitors, and activators etc., and to evaluate the biological roles of the proteins \textit{in vivo}. Once a structural model is available, site-directed mutagenesis of specific residues is often undertaken to determine the protein's structure/function relationships in detail.

Ala scanning is a commonly used mutagenesis approach, in which protein residues of interest (i.e., in the active site, ligand binding site or protein interface) are one-by-one systematically changed from their identity in the wild-type protein to Ala in a corresponding mutant. This is useful to create mutant proteins, but the effects of specific amino acid substitutions are not generally predictable. Another approach is to randomly mutagenize a codon or group of codons by random substitution of nucleotides; the group of mutants can then be subjected to genetic selection (12, 13). If a particular property of a side chain (length, hydrophobicity etc.) is important at a given position, only side chains with conservative amino acid substitutions will be allowed in functional mutants (i.e., those that survive the selection process). This method reinforces the interpretation of X-ray crystal structures and helps to define the functional roles of each amino acid residue \textit{in vivo}. 

Such mutagenesis studies have exclusively used *Escherichia coli* as the host for expression and selection of mutant proteins. Large numbers of mutant proteins are easily analyzed in *E. coli*; however, high molecular weight mammalian enzymes that are disable to be expressed in *E. coli* can not be analyzed by this approach in this host. In the present study, random mutagenesis was carried out on human topoisomerase 2α (TOP2α). In order to characterize mutants of TOP2α, they were expressed in a yeast strain carrying a temperature-sensitive endogenous yeast TOP2, which can be genetically complemented by the gene encoding wild-type human TOP2α. This mutagenesis study specifically targets amino acid residues 793-808 around the active site of human TOP2α. This highly conserved region is of particular interest because it is involved in both catalytic activity and drug sensitivity. The results presented here provide useful information on the roles of these active site residues. Based on the amino acid substitutions allowed in active mutants and their drug sensitivity profiles, possible functions of the targeted amino acid residues are proposed and discussed.

**Experimental Procedures**

**Strains, cDNA and oligonucleotides** -- The yeast strain SD1-4 (MATa, ade1, ade2, ura3-52, top2-1) was kindly provided by Dr. S. DiNardo, State University of New York, and JN394t2-4 (MATa, ura3-52, leu2, trp1, his7, ade1-2 ISE2, rad52::LEU2 top2-4 ) by J. Wang, Harvard University. A full length of human TOP2α cDNA was also from J. Wang. *E.coli* DH5α [deoR, endA1, gyrA96, hsdR17(rk- mk+), recA1, relA1, supE44, thi-1, Δ(lacZYA-argFV169), Φ80δlacZM15, F-, λ-] was used for cloning and plasmid constructions. DNA oligomers were synthesized and purified by Amersham Pharmacia Biotech (Buckinghamshire, UK) or NK products (Osaka, Japan).
**Plasmid constructions** - The short *XbaI-HindIII* fragment of pYES2 (Invitrogen, Groningen, Netherlands) was replaced with a synthetic oligomer 5’-CTA GGC TCG AGA TGC TAC GTA AGT CAG CCC GGG CTG CGG CCG CT, which is annealed with 5’-AGC TAG CGG CCG CAG CCC GGG CTG ACT TAC GTA GCA TCT CGA GC (LLpYES-PGAL1). LLpYES-PGAL1 contains a new restriction site for *NotI* and lacks *XbaI* and *KpnI* sites. The full length of human TOP2α cDNA was transferred into the large *NotI-XhoI* fragment of LLpYES-PGAL1 (LLpYES hTOP2-PGAL1).

Non-functional Dummy TOP2α was constructed by insertion of a synthetic oligomer 5’-GTT ACC ATC GCA TGC AAG CTT GCT CAG C (DUM-U) annealed with 5’-GCT GAG CAA GCT TGC ATG CGA TGG TAC C (DUM-D) in the large *HpaI-KpnI* fragment of PSL1190hTOP2. The *NotI* - *XhoI* fragment was further replaced with the corresponding site of LLpYES hTOP2-PGAL1 (LLpYES hTOP2DUM-PGAL1, or Dummy Vector).

**Random oligonucleotides** - An equimolar mixture of random oligonucleotides was made from 16 synthetic oligomer pools with substitutions at codons 788-813 of human TOP2α, 5’-GGT CAG TTT GGT ACC (AGG CTA CAT GGT GGC AAG GAT TCT GCT AGT CCA CGA TAC ATC TTT ACA) ATG CTC AGC TCT TTG. In each primer, either one of the target codons, Arg793-Thr808, shown in parentheses in the above sequence, was randomized by including all 4 nucleotides in equimolar amounts at the appropriate synthetic cycle. We also introduced a silent mutation at a codon downstream of each target for 15 out of 16 random oligomers (Arg793-Phe807). Two silent mutations in Arg793 and His795 were placed in the random oligomer that targeted residue Thr808. For example, the Arg793 random oligomer had the sequence 5’-GGT CAG TTT GGT ACC NNN CTg CAT GGT GGC AAG GAT TCT GCT AGT CCA CGA TAC ATC TTT ACA ATG CTC AGC TCT TTG, where the N represents an equimolar mixture of the G,C,A and T, and the small letter "g" denotes a silent mutation. The silent mutations were used to identify the origins of the
oligomer when the wild-type sequence was restored during synthesis. As a result, the random oligonucleotides theoretically contain equimolar amount of the 1024 possible mutations as single amino acid substitutions.

**Library construction** - To construct a human TOP2α mutant plasmid library, the mixture of random oligonucleotides was used as a forward PCR primer. A reverse primer was designed complementary to the XbaI site of hTOP2cDNA sequence (5'-CAT GGG TTC TAG AAC TTG TTC). Using PSL1190hTOP2, as a template, 25 cycles of PCR (94 oC for 30 sec, 45 oC for 30 sec, 72 oC for 30 sec) were carried out using the proofreading-competent Pyrobest DNA polymerase (TaKaRa, Kyoto, Japan) with appropriate buffers. The 380 bp PCR product was isolated by electrophoresis in a 0.8% agarose gel and purified by QIAEXII GEL EXTRACTION KIT (QIAGEN, Valencia, CA). This fragment was treated with restriction enzymes KpnI and XbaI, and purified again by the same procedure (Randomized Cassette). In some experiments, one of 16 random oligomers was used as a primer for library construction (see below).

The KpnI - XbaI large fragment of the LLpYES hTOP2DUM-PGAL1 and the Randomized Cassette were ligated and used for transformation of DH5α. An aliquot of the transformed *E.coli* was plated on 2XYT [16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl (pH 7.3)] containing carbenicillin (100 µg/ml) to determine the total number of transformants, and the remainder was inoculated into 500 ml 2XYT and cultured at 37 oC overnight. Plasmids were recovered and used as a mutant plasmid library. The random library consists of approximately 6000 independent clones.

**Complementation assay** - The yeast strain SD1-4 was used for transformation with the human TOP2α mutant plasmid library by Frozen-EZ Yeast Transformation II (ZYMO RESEARCH, Orange, CA). The transformants were cultured on solid medium, SD-URA
(6.7 g/L yeast nitrogen base w/o amino acids, 5 g/L casamino acids, 20 g/L glucose, 20 mg/L adenine sulfate, 20 mg/L tryptophan, 15 g/L Bacto agar) at 25 °C for 48 h, replicated on a plate containing SDGAL-URA by using Replica Plater (TaKaRa, Kyoto, Japan), in which glucose of the SD-URA medium was replaced by 20 g/L galactose. The replicated colonies were cultured at 37 °C for 7 days. Colonies were picked and cultured in tubes with 2 mL SD-URA at 25 °C overnight. The yeast cells were collected, resuspended in 200 µL of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM Na2EDTA), and lysed by vigorous mixing for 10 min with 300 mg acid-washed glass beads (300 micron, SIGMA, St. Louis, MO) and 200 µL of Phenol CIA (phenol: chloroform: isoamyl alcohol = 25: 24: 1, mixed pH 6.7, Nacalai tesque, Kyoto, Japan). After centrifugation and ethanol precipitation, the purified DNA was used for transformation of DH5α by electroporation according to BIO-RAD (Richmond, CA). The transformed cells were incubated on 2XYT plate containing carbenicillin (100 µg/mL) at 37 °C overnight. Colonies were picked up and inoculated into 2 mL of 2XYT solution containing carbenicillin (100 µg/mL) at 37 °C overnight. Plasmids were extracted and purified by Wizard Plus Mini Preps DNA purification system (Promega, Madison, WI), and used for a second transformation of the SD1-4 strain to confirm colony formation.

**DNA sequencing** - The target regions of active mutants were sequenced using the Taq Dye Terminator Cycle Sequence Kit, with a PE -Applied Biosystems 373A DNA Sequencer (Foster City, CA). An oligomer, 5’-GACAGTGGTGAAATGT, was used for the sequencing primer.

**Complementation assay using the independent libraries** - Construction of independent libraries and complementation assays were carried out using the same procedures as described above, except that each single random oligomer was used for the library construction. Each library contains 64 codon variations at the targeted position.
**Site directed mutagenesis** - *Cel II* restriction sites outside the TOP2α gene of the Dummy Vector were knocked out by a *Cel II* partial restriction, treatments with Klenow fragment and T4 ligase. Oligomers between the *Kpn I* and *Cel II* sites of human TOP2α with desired mutations were chemically synthesized and used for replacement with the corresponding fragment of the Dummy Vector.

**Etoposide resistance assay** - To investigate etoposide resistance, a permeable yeast strain JN394top2-4 was transformed by the functional mutants of human TOP2α by means of electroporation (GENE PULSER II, Richmond, CA). After 7 days of culture on SD-URA plates at 25 oC each transformant was picked and streaked on SDGAL-URA plate with or without etoposide (100 µg/mL, SIGMA, St. Louis, MO). Etoposide resistance was determined after culture at 35 oC for 7 days.

**Results**

**Construction of yeast expression library for mutants of human TOP2α** - The primary and secondary structures of the active site region of TOP2 are conserved among species from bacteria to human [Fig. 1 and references (3, 4, 5)] and are essential for catalysis (1). The active site includes a critical tyrosine residue that becomes covalently linked to DNA during catalysis (Tyr805 in human TOP2α). In *Saccharomyces cerevisiae* (*S. cerevisiae*) TOP2, this active site Tyr residue is located at the C-terminal edge of a loop structure on the surface of the A' domain (4). In order to study the functions of individual residues in this region of human TOP2α, random substitutions were made for amino acids 793-808 and mutants with enzymatic activity were selected by genetic complementation (Fig. 2).
Sixteen oligonucleotide pools were synthesized that are complementary to the 78 bases around the TOP2 active site. Each oligonucleotide pool contains randomized nucleotides at one of the 16 codons in amino acids 793-808. A 380 bp fragment of the TOP2 gene was amplified using a mixture of these 16 "mutant" oligonucleotide pools as one PCR primer and a "wild-type" oligonucleotide as the other PCR primer. The PCR product was purified, digested by KpnI and XbaI, and used to replace the same restriction fragment of the Dummy Vector (see Experimental Procedures). In theory, the library of plasmids recovered from ligating the PCR product with the Dummy Vector direct synthesis of wild-type human TOP2 and 19 single amino acid substitution mutants at each residue in the targeted region in human TOP2α. However, the representation of different mutants in the library depends on the efficiency of synthesis of each mutant oligonucleotide and the efficiency with which it is used in the PCR reaction. To test the actual composition of the library, 49 clones were arbitrarily selected and their DNA sequences were determined (Fig. 3). All theoretically possible mutations were found within the randomized region, and all isolates had single amino acid substitutions at 13 positions out of the 16. Although in 5 cases the same substitutions were recovered twice, each contained different nucleotide sequences with a few exceptions (i.e., two Leu substitutions at Asp799 and two Gly substitutions at Ser802).

**Genetic selection of functional human TOP2α mutants** - The temperature-sensitive *S. cerevisiae* strain SD1-4 (*top2-1*) was used to select active variants of TOP2 from the library of mutants by *in vivo* complementation assay. Previous studies have demonstrated that the temperature sensitive phenotype in yeast can be complemented by transformation with a plasmid that expresses either mouse (6) or human TOP2 (14). A plasmid that expresses wild-type human TOP2α also suppresses the temperature-sensitive phenotype of SD1-4 at 37 °C, although a plasmid expressing an inactive human TOP2α (i.e., Y805L) or the vector alone, does not complement the host strain (Fig. 4).
In vivo selection was carried out to identify functional human TOP2α mutants in the plasmid library; however, accurate discrimination between functional and non-functional mutants requires a sensitive and strict complementation specificity. To test this system using SD1-4, cells were screened at 37 °C after being transformed with plasmid DNA mixtures containing wild-type human TOP2-expressing plasmid and the Dummy Vector at the following ratios: 100: 0, 50: 50, 25: 75, or 0: 100. Colonies were seen on plates only when the mixture included the wild-type vector. Plasmid DNA was isolated from 20 individual clones from each transformation and their sequences were determined. All plasmids from the 100:0 mixture, and 19 of 20 from the 50: 50 and 25: 75 mixtures were wild-type plasmids. Thus, the system provides a way to discriminate the non-functional clone when a homogeneous plasmid was used for transformation, but the rate of false positives is 5% when a mixed population of input DNA is screened. To completely exclude non-functional mutants in the subsequent experiments, each plasmid recovered from the first round of selection was subject to a second round of selection in the same strain. After the second round of selection, the false positive clones were effectively removed.

Active mutants of human TOP2α - This random library was transformed into yeast and 200 independent transformants were grown at 30 °C (permissive temperature). Colonies were transferred to fresh plates by replica plating and cultured for a week at 37 °C (restrictive temperature). Approximately half of the colonies that grew at 37 °C also grew at 30 °C. A total of 108 plasmids were isolated from the temperature-resistant colonies, and the DNA sequence of the mutated region was determined. Six of the 108 plasmids unexpectedly contained double mutations, and these were not studied further. Four mutants did not form colonies when the plasmid was retransformed and were regarded as false positives. As a result, 98 clones encoding active mutants of human TOP2α were obtained for further study.
Among the 98 active mutants of TOP2α selected by this screen, there were 83 missense and 15 silent mutations. Some of the missense substitutions were nonredundant at the nucleotide or amino acid level, so that the pool of active TOP2 mutants represented 68 different codon substitutions and 50 different amino acid substitutions in the targeted active site region (Fig. 5).

The amino acid substitutions were not random. Leu798, Ser802 and Pro803 were among the residues that were most tolerant of variation; active mutants were selected in which these residues were replaced by amino acids with variable charge and side chain lengths. In contrast, Leu794, Gly797, Asp799, Ala801, Arg804, Tyr805, Ile806, Phe807 and Thr808 tolerated only subtle changes (Fig 5B), and tended to be unchanged or were replaced only by very similar amino acids (conservative changes); however, silent mutations were found in the codons for Arg804 and Phe807 (Fig. 5A).

Active mutants in independent libraries - Amino acids that allow only limited substitution without resulting in loss of enzyme activity may play a strictly defined role in that protein's function (12, 13, 15). Although the initial screen described above identified 50 allowable amino acid substitutions in active variants of human TOP2α, it was not a comprehensive screen of such mutants. Using a mixture of the 16 random cassettes as a library source, thousands of positive clones would have to be screened, isolated and sequenced in order to make statistically valid conclusions about all possible active variants in the library. Therefore, 9 additional independent libraries were prepared, each of which included random substitution at the codon for one of the following amino acids: Leu794, Gly797, Asp799, Ala801, Arg804, Tyr805, Ile806, Phe807 and Thr808. Complementation experiments were carried out until at least 200 transformants were screened from each library; this number is >3 times higher than the number required to screen one copy of each possible substitution at a target codon (Fig. 6).
All active clones isolated from the library of mutants at Tyr805 library had a Tyr residue at the targeted position (Fig. 6B). Because a Dummy Vector was used to construct these libraries, wild type clones can not be contaminated by incomplete restriction digestion. Therefore, the active variants with a Tyr codon at 805 were generated from the randomly substituted oligonucleotide pool. In the Tyr805 random mutant library, it is predicted that 6.3 Tyr codons would be found among the 200 colonies screened (assuming the library contains all codons in equimolar proportions). Consistent with this prediction, 6 wild-type clones were identified by screening the Tyr805 mutant library (Fig. 6A).

Among the other amino acids studied, four positions allowed only conservative substitutions to conserve enzyme activity (16). Leu794 could be substituted with Ile or Val, the other two aliphatic side chains. Asp799 could be substituted with the acidic side chain of Glu, Ala801 with the small amino acids Gly and Ser, and Arg804 with the basic amino acid Lys (Fig. 6B). These data show that Tyr805 is immutable, and that Leu794, Asp799, Ala801 and Arg804 are nearly immutable residues of human TOP2α, as detected by this genetic complementation assay.

**Mutants that are resistant against etoposide** - By screening these 9 libraries of mutants at individual codons in the active site of human TOP2α, 66 functional mutants of human TOP2α were identified in total. Because these substitutions were introduced around the active site, these mutants are likely to include enzymes with altered catalytic specificities and properties, such as altered drug sensitivity. This possibility was tested by transforming the mutant plasmids into the JN394t2-4 strain, and plating the transformants on agar plates containing 100 µg/mL etoposide. Three mutants were selected as etoposide-resistant clones. One mutant, Pro803Ser, showed a weak resistance to etoposide, as reported previously (7, 17). The other mutants, Lys798Leu and Lys798Pro, also formed etoposide-resistant
colonies. The relative resistance of these mutants were in the order K798P > K798L > P803S (Fig. 7).

**Discussion**

*Tolerance to amino acid substitutions in the genetic complementation* – We have introduced single amino acid substitutions into the 16 amino acids around the active site of the enzyme, and these mutant enzymes were used to complement a TOP2 temperature-sensitive mutation in *S. cerevisiae*. Tolerance to amino acid substitutions at each residue was determined by using libraries including either a mixture of mutants at the 16 targeted amino acids or including mutants at only one of the 16 targeted amino acid residues (Fig. 5, 6).

The results of the complementation assay suggest that the targeted amino acids can be categorized into 4 groups. The first group includes only one member, the active site Tyr805. This Tyr residue is involved in transesterification to the DNA phosphodiester bond; no active mutant could be isolated with any substitution in this position in experiments with the mixed codon and the single codon targeted mutant libraries. As shown in Fig. 3, the plasmid libraries include variants that synthesize proteins with amino acid substitutions at these positions; however, these non-functional substitutions do not complement the temperature sensitive yeast host strain. Such mutants were also reported previously, and were created by site-directed mutagenesis at this site. For example, a DNA topoisomerase IV mutant that has a conservative substitution of His for Tyr805 carries out nicking of single-stranded DNA, but does not carry out double-stranded DNA cleavage (18). That study agrees with the results presented here, in that it also demonstrates a strict requirement for a Tyr residue at position 805 in order to retain TOP2 activity.
The second group of residues identified in this mutant screen includes Leu794, Asp799, Ala801, and Arg804; these four residues were susceptible to conservative amino acid substitutions. Leu794 can be substituted with other aliphatic amino acids. Asp799 with another acidic side chain Glu, Ala801 with amino acids Gly and Ala that are small, and Arg804 with another basic side chain Lys (Fig. 6B). These amino acids play such important roles in enzyme function that only extremely limited substitutions can occur without loss of catalytic efficiency. Arg804 is immediately adjacent to the active site Tyr805. In DNA gyrase, a similar set of amino acids has been proposed to form the active site of the breakage-reunion reaction (5). In addition, Arg781 from S. cerevisiae TOP2, which corresponds to Arg804 in the human enzyme, has been subject to site-directed mutagenesis (19). The results of that study suggest that Arg781 might be involved in anchoring the 5' side of the broken DNA (19). Our results are consistent with those results, and also demonstrate that another basic residue, Lys, could substitute for Arg at this position, although Arg is strictly conserved in naturally occurring proteins from various species (Fig. 1). Interestingly, in this group of four residues that allow conservative substitutions, Ala801 and Arg804 are highly conserved in all species including prokaryotes, while Leu794 and Asp799, that are located farther from the active site, are conserved only in higher eukaryotes in the primary structure.

The last two categories of residues in the active site region of human TOP2α are much more tolerant of amino acid substitution. Ile806 and Phe807 are tolerant to all substitutions except those with charged side chains; and the last group, His795, Lys798, Ser800, Ser802, Pro803, and Thr808, are tolerant to a variety of amino acid substitutions including alterations in side chain length, hydrophobicity and charge. Gly796 is tolerant of various substitutions, and belongs either in the third or fourth groups. Residues in the third or fourth group are not essential for TOP2 function. Nevertheless, it is surprising that these residues have not randomly drifted in the course of evolution (Fig. 1), but instead are represented by a rather narrow ranges of amino acids in naturally occurring TOP2 sequences. On explanation for
this is that some substitutions at these positions might have little or no direct effect on enzyme activity, but may alter other characteristics of the enzyme, such as its sensitivity to environmental stress, that are not evident in the screen carried out here.

**Etoposide resistant mutants** - It was somewhat surprising to find that Lys798 was highly mutable by this screening procedure. It has been proposed that the corresponding residue in *S. cerevisiae* TOP2 interacts with the single-stranded region of the substrate DNA (19, 20). If this interaction were to be essential, it would be predicted that Lys798 would be relatively immutable. One possible explanation is that Lys798 is involved in an interaction with DNA, but that this interaction is dispensable for efficiency of the catalytic functions. Such interactions have been described in *Taq* DNA polymerase I. For example, Thr664 in this polymerase interacts with the template DNA in the closed ternary complex (21) and was highly mutable in a genetic complementation assay (15). However, the substitution was not totally without effect on the enzyme, because it decreased the fidelity in DNA replication (22). If a comparable situation applies to Lys798 of TOP2, then some mutants of Lys798 may have altered biochemical properties which have not been identified in this study.

Support for the above hypothesis was found by testing the active variants of TOP2α identified in this study for drug resistance. Among the 3 etoposide resistant mutants that were identified, two mutants had amino acid substitutions of Leu or Pro at Lys798 (K798L and K798P, Fig. 7). These mutants also exhibited the resistance in an *in vitro* experiment using overexpressed and purified proteins (manuscript in preparation). Another mutant at this position, K798N, has been described previously; this mutation occurred during selection of a cloned human leukemic cell line (23). However, the relationship between the substitution and drug resistance has not been proven for K798N.
Implication for a structural model of TOP2α - The amino acid sequence of human TOP2α is nearly 50% homologous to the sequence of *S. cerevisiae* TOP2 (3). Furthermore, human TOP2α is functionally interchangeable with *S. cerevisiae* TOP2 in the yeast cell [this study and (14)]. These results suggest that these two proteins share a common conformation at the level of tertiary structure, especially around the active site [i.e., at the CAP-like folds; see (4, 24, 25)]. Based on this assumption, the functional data presented here was superimposed on structural information for *S. cerevisiae* TOP2 (4).

Fig. 8 shows the locations of human amino acid residues Arg793-Thr808 on a schematic diagram of the *S. cerevisiae* TOP2 polypeptide backbone. Side chains are shown in four colors as categorized above: red, purple, yellow, and light blue, for groups 1 (immutable), 2 (conservative substitutions only), 3 (highly mutable, but not to charged side chains) and 4 (highly mutable), respectively. In *S. cerevisiae* TOP2, the first three targeted amino acids are in the α5-helix (Fig. 8A). This structure may also exist in human TOP2α, because the corresponding region (Arg793 - His795) is located between the three conserved Glycines at 791, 796 and 797 in both human and *S. cerevisiae* enzymes. Ile806 and Phe807 could not be substituted with any hydrophilic side chains, which may indicate that they are buried towards the hydrophobic interior of the molecule (26, 27). In agreement with this observation, in *S. cerevisiae* TOP2, the corresponding amino acids extend toward the inside of the protein (β3 sheet, Fig. 8A). Thus the first three and last three residues of the target loop (Fig.1) of human TOP2α superimpose well on the yeast TOP2 structure (Fig.8).

In wild-type human TOP2α, two Gly residues and a Pro residue are found between Gly796 and Pro803. These two amino acids are not favored to form either α-helix (28, 29, 30) or β-sheet (31, 32). The number of Gly and Pro residues can be further increased by substitutions without loss of activity (K798G, K798P, and S802G). Therefore, this region may form a random coil structure in human TOP2α, just as seen in *S. cerevisiae* TOP2.
Charged residues Lys798, Asp799 and Ser802, which was substituted by Arg (Fig. 5B), must be exposed on the surface of the protein.

The four amino acids (Leu794, Asp799, Ala801, Arg804) and the active site Tyr805 must be functionally important because they are well conserved or unchangeable, and because they form a plane on the polypeptide backbone (Fig. 8B). In the crystal structure of DNA gyrase A, Morias Cabral et al. modeled a double-stranded DNA substrate on the surface of the protein, and found that the active Tyr residues of each GyrA subunit, located approximately 27 Å apart, must move to achieve appropriately staggered positions for cleavage of the 5' ends of the duplex (5). In DNA gyrase A, the side chain positions of Ala118, Arg121, and Tyr122 can be superimposed on the corresponding positions of Ala801, Arg804 and Tyr805 (data not shown). Interestingly, Leu794 and Asp799 of human TOP2α also come very close to Ile112 and Asp115 of DNA gyrase A, respectively, in the same structure (data not shown). Therefore, It would be an attractive picture that this plane, including the active Tyr, performs catalysis through the conformational change in TOP2α. According to this hypothetical model, the DNA molecule is held, cut and rejoined on this planar surface.

The etoposide-resistant clones identified in this study may support this model. In bacterial DNA gyrase that are sensitive to synthetic quinolone compounds (33, 34), resistant mutants have been mapped near the putative interaction site with DNA (5). Human TOP2α is inhibited by etoposide through formation of the cleavable complex (35), like bacterial DNA gyrases by quinolone compounds. Therefore, amino acids that are associated with etoposide-sensitivity might come close to the DNA substrate in this region.

Our model is also consistent with the co-crystal structures of proteins that share the winged-helix motif with TOP2 (5, 24). In the HNF-3/fork head DNA recognition motif
(36), a part of the wing (W1) of HNF-3, which may be structurally homologous to the target loop structure in human TOP2α, is considered to interact with DNA. The loop stem is stabilized by β-sheet hydrogen bonding and side chain-backbone interactions.

In the absence of any binary structures of human TOP2α and the substrate DNA, however, we do not rule out the other possibilities. For example, an alternative explanation is that the plane is involved in the interaction with the B’ domain, although this is less likely because such amino acids may tolerate nonconservative substitutions (37). More plausible story is that some of the conservative amino acids are required for the efficient conformational changes during the catalysis, or critical in keeping random coil structure of the loop region.

**Acknowledgments**

This work is supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to AK, YN and SY, and the Nitto Foundation to MS. The authors thank J. Wang for helpful discussion and for providing the co-ordinates of the putative TOP2-DNA interactions (19). We are also grateful to Tazuko Tomita for technical assistance.
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Figure Legends

Fig. 1. **Amino acid alignment of the active site region of TOP2.** Amino acid sequences of topoisomerase II from various species were obtained from EMBL. Number at the top of the figure represents the amino acid number of human TOP2α from 793 (Arg) to 808 (Thr), corresponding to that from 771 to 786 in *S. cerevisiae*, and to that from 110 to 125 in *E. coli*. The abbreviations used are: Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tryp, *Trypanosoma brucei*; Cfa, *Crithidia fasciculata*; GyrA, gyraseA; Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Bs, *Bacillus subtilis*; Mp, *Mycoplasma pneumoniae*; Sa, *Staphylococcus aureus*.

Fig. 2. **Selection strategy.** The random oligonucleotide mixture and a reverse primer were designed for amplifying the 380 bp fragment of TOP2 by Pyrobest DNA polymerase. This randomized cassette was used to replace the corresponding region of the Dummy Vector. The plasmid library, containing 6000 independent mutant clones, was transferred into the temperature sensitive yeast strain SD1-4. Other conditions were described in Experimental Procedures.

Fig. 3. **Mutations in the mixed random library in *E.coli*.** Twenty-five samples from the mixed random library in *E.coli* DH5α were cloned and sequenced. The amino acid positions of human TOP2α are indicated at the top. All substitutions represent single amino acid replacements. Silent substitutions are listed in italic. When the same amino acid substitution was isolated more than once, the number of isolates is also indicated.

Fig. 4. **Functional complementation of a TOP2<sup>ts</sup> yeast strain SD1-4 by human TOP2α.** *S. cerevisiae* SD1-4 (*top2-1*) was transformed with either a vector pYES, LLpYEShTOP2DUM-PGAL1 that carries inactive TOP2α, LLpYEShTOP2-PGAL1 that
carries wild-type human TOP2α, or LLpYES-PGAL1-Y805L that carries a mutant TOP2α at the active center. Transformed cells were streaked on an SD-URA plate and incubated at 25 °C (A) and on SDGAL-URA plate at 37 °C (B). In the center of the panels, names of the clones are indicated: LpYes, pYES2-PGAL1; Dummy, LLpYES hTOP2DUM -PGAL1; WT, LLpYEShTOP2-PGAL1; Y805L, Leu substitution at Tyr805. Open arrow head indicates the wild-type culture.

Fig. 5. **Compilation of amino acid substitutions using the mixed library.** SD1-4 yeast were transformed with the mixed random libraries. The DNA sequence of the complementing clones are indicated in triplet codons (A) and deduced amino acid residues (B). The number of amino acid positions is as indicated in Fig. 3. Solid triangles indicate the amino acid residues where either no substitution or only conservative amino acid substitutions were found. All substitutions were single amino acid replacements. In A, the number of substitution at each amino acid is listed under the wild type sequence. Codons and the corresponding amino acids are at the left. Silent substitutions are shown in italic. In B, substituted amino acid residues at each site are listed. Silent substitutions are not listed. When the same amino acid substitution was isolated more than once, the number of isolates is also indicated.

Fig. 6. **Compilation of amino acid substitutions using the independent libraries.** SD1-4 yeast was transformed using the independent random libraries for Lys794, Gly797, Asp799, Ala801, Arg804, Tyr805, Ile806, F807, and Thr808. Clones that complemented the temperature-sensitive growth were sequenced. The nucleotide sequences are shown in triplet codons (A) and deduced amino acid residues (B) as in Fig. 5. Solid triangles indicate the amino acids of interest. G797E and R804K were also constructed by site-directed mutagenesis and their activity was confirmed by independent complementation. Based on the number of screened transformants and silent substitutions,
all the possible amino acid substitutions were surveyed with a probability greater than 95 %.
Some codons might not be isolated due to codon usage preference in S. cerevisiae
(ftp://genome-ftp.stanford.edu/pub/codon/ysc.orf.cod).

Fig. 7. **Assignment of etoposide resistance in yeast strain JN394t2-4.** Wild-type and mutants in human TOP2α (K798P, K798L, K798C, P803S) were used to transform JN394t2-4 (*ISE2, top2-4*). Each clone that carries either wild-type or mutant TOP2α was cultured and streaked on an SDGAL-URA plate with (A) or without (B) 100 µg/mL etoposide (VP16). Drug sensitivity was determined after incubation at 35 °C for 1 week. In the center of the panels, names of the clones are indicated. P803S is indicated by an open arrow head, and K798P and K796L are indicated by filled arrow heads.

Fig. 8 **Structural model of human TOP2α.** A. The winged motif of the A' subdomain in the *S. cerevisiae* crystal structure (AA702-AA792) is shown with the side chains that belong to group 1 (red) and the group 2 (purple). Two amino acid residues that are involved in etoposide sensitivity are also shown (black). The polypeptide backbone between the Arg793-Thr808 is highlighted in gray, and the remaining region is in green. The names of α5-helix β3-sheet, N-terminal site (N), and some of amino acids are indicated in the figure. B. The target loop is shown in a stereo view with all side chains. In both panels, side chains are replaced with the corresponding human amino acid residues, and referred to as the human positions (Arg793-Thr798). The coordinate sets were obtained from the Protein Data Bank (38). The drawing was made by using the program package InsightII (Molecular Simulations Inc, San Diego, CA). The polypeptide backbone between the Arg793-Thr808 is shown as a gray ribbon. Side chains: red, group 1 (Tyr805); purple, group 2 (Leu794, Asp797, Ala801, and Arg804); yellow, group 3 (Ile806 and F807); light blue and black, group 4 (Arg793, Lys798, Ser800, Ser802, and Thr808). Among the group 4 side chains, two that were involved in etoposide sensitivity are shown in black (Lys798 and Pro803).
### Fig. 1

|    | 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Eukaryote** |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| human TOP2α | R   | L   | H   | G   | G   | K   | D   | S   | A   | S   | P   | R   | Y   | I   | F   | T   |
| human TOP2β | R   | L   | H   | G   | G   | K   | D   | A   | A   | A   | S   | P   | R   | Y   | I   | F   | T   |
| *Dm* TOP2    | R   | L   | S   | G   | G   | K   | D   | C   | A   | S   | A   | R   | Y   | I   | F   | T   |
| *Sc* TOP2    | R   | A   | T   | G   | G   | K   | D   | A   | A   | A   | A   | A   | R   | Y   | I   | N   | T   |
| *Sp* TOP2    | R   | S   | E   | G   | G   | K   | N   | A   | S   | A   | S   | R   | Y   | L   | F   | T   |
| *Tryp* TOP2  | R   | Q   | Q   | L   | G   | N   | D   | H   | A   | A   | P   | R   | Y   | I   | F   | T   |
| *Cfa* TOP2   | R   | Q   | Q   | L   | G   | N   | D   | H   | A   | A   | P   | R   | Y   | I   | E   | T   |
| **Prokaryote** |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| *Ec* GyrA    | G   | S   | I   | D   | G   | D   | S   | A   | A   | A   | M   | R   | Y   | T   | E   | I   |
| *Kp* GyrA    | G   | S   | V   | D   | G   | D   | S   | A   | A   | A   | M   | R   | Y   | T   | E   | I   |
| *Ba* GyrA    | G   | S   | V   | D   | G   | D   | S   | A   | A   | A   | M   | R   | Y   | T   | E   | A   |
| *Mp* GyrA    | G   | S   | I   | D   | G   | D   | R   | P   | A   | A   | Q   | R   | Y   | T   | E   | A   |
| *Sa* GyrA    | G   | S   | M   | D   | G   | D   | G   | A   | A   | A   | M   | R   | Y   | T   | E   | A   |
Fig. 2

randomized oligonucleotides

hTOP2α cDNA

PCR

randomized cassettes

hTOP2α cDNA

Dummy

hTOP2α mutant plasmid library
Fig. 3

| 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| R:2 | L Y A A:2 D A A G:2 C:2 C S I |
| Y G A I L A F L I S:2 L V D |
| stp V L R G T stp:2 N A |
| T G S L K |
|       V |
|       L:2 |
Fig. 4

A

SD-URA 25 °C

B

SDGAL-URA 37 °C
### Fig. 5A

| AA codon | 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          | GCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          | GCG |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          | GCT |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| C        |     | TGC |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     | TGT |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     | TTC |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| F        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| I        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| L        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| M        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| P        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Q        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| S        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| T        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| V        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| W        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Y        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| H        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| R        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| D        |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|          |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| R   | L   | H   | G   | G   | K   | D   | S   | A   | S   | P   | R   | Y   | I   | F   | T   |
| G:2 | G:2 | L   | G:3 | C:2 | S:2 | R:6 | S:5 | L   | Y   |     |     |     |     |     |     |
| V:2 | A   | V   | V:3 | D   | L:4 | V:5 |     |     |     |     |     |     |     |     |     |
| A   | I   | Y   | P:2 | F   | T:2 | L:4 |     |     |     |     |     |     |     |     |     |
| C   | L   | R:2 | L   | V:2 | G:3 |     |     |     |     |     |     |     |     |     |     |
| I   | C   | M   | C   | C:2 |     |     |     |     |     |     |     |     |     |     |     |
| S   | L   | P   | F   | A   |     |     |     |     |     |     |     |     |     |     |     |
| W   | M   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|     | S   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

Fig. 5B
![Fig. 6A](http://www.jbc.org/)

| AA codon | 793 | 794 | 795 | 796 | 797 | 798 | 799 | 800 | 801 | 802 | 803 | 804 | 805 | 806 | 807 | 808 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|           | R   | L   | H   | G   | G   | D   | S   | A   | S   | P   | Y   | I   | F   | T   |
| A         | GCA | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GCC | 7   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GCT | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| F         | TTC | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TTT | 4   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GGA | 4   | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GCC | 7   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GGC | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GGG | 4   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| G         | GGT | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| T         | ATA | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | ATC | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | ATT | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| L         | TTA | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TTG | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CTA | 6   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CTC | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CTG | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CTT | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| M         | ATG | 4   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| V         | GTC | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | GTT | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Q         | CAA | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CAG | 2   | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| S         | AGT | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TCA | 2   | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TCC | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TCG | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TCT | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| T         | ACA | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | ACC | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | ACG | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | ACT | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Y         | TAC | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | TAT | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| K         | AAG | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| R         | AGA | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | AGG | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CGC | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CGG | 8   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | CGT | 7   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| D         | GAC | 2   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | GAT | 8   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| E         | GAA | 3   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|           | GAG | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
Fig. 7

A  
SDGAL-URA

K798C  
P803S  
K798L  
K798P

B  
SDGAL-URA

Etoposide 100 µg/ml
Fig. 8A

- R804
- Y805
- A'β3
- A801
- P803
- D799
- L794
- A'α5
- K798
- N
Assignment of functional amino acids around the active site of human DNA topoisomerase II alpha
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J. Biol. Chem. published online May 11, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003243200

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