Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3653–3657, April 1996
Biochemistry

Human *TOP3*: A single-copy gene encoding DNA topoisomerase III

(sequence homology/chromosomal location/relaxation of supercoiled DNA/genome stability)

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ABSTRACT **A human cDNA encoding a protein homologous to the *Escherichia coli* DNA topoisomerase I subfamily of enzymes has been identified through cloning and sequencing. Expressing the cloned human cDNA in yeast Δ*top1* cells lacking endogenous DNA topoisomerase I yielded an activity in cell extracts that specifically reduces the number of supercoils in a highly negatively supercoiled DNA. On the basis of these results, the human gene containing the cDNA sequence has been denoted *TOP3*, and the protein it encodes has been denoted DNA topoisomerase III. Screening of a panel of human–rodent somatic hybrids and fluorescence *in situ* hybridization of cloned *TOP3* genomic DNA to metaphase chromosomes indicate that human *TOP3* is a single-copy gene located at chromosome 17p11.2–12.**

In eukaryotes, enzymes belonging to each of the three subfamilies of DNA topoisomerases have been identified. Eukaryotic DNA topoisomerase I catalyzes the removal of positive and negative supercoils by transiently cleaving one DNA strand for the passage of another, and is thus classified as a type I topoisomerase (1). The enzyme appears to be the major cellular activity in the removal of supercoils generated by processes such as replication and transcription (2–4). *Top1* null mutants of the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* are viable (2, 3), and various experiments suggest that the dispensability of the enzyme is due to substitution of its cellular function by DNA topoisomerase II (5–7). In *Drosophila*, however, DNA topoisomerase I is essential, probably because of the rapid rate of DNA replication during early embryogenesis (8).

Eukaryotic DNA topoisomerase II is a type II topoisomerase, which catalyzes the transient breakage of a double-stranded DNA and the transport of another DNA double helix through the break before its resealing (9–11). This enzyme catalyzes both the removal of positive and negative supercoils and the unlinking of intertwined pairs of DNA double helices. The latter activity cannot be substituted by type I topoisomerases, and thus a type II DNA topoisomerase is essential for disentangling DNA during condensation and mitotic and meiotic segregation of chromosomes (2, 3, 12–16).

Eukaryotic DNA topoisomerase III was discovered in 1989 (17). The gene encoding the yeast enzyme was originally identified by its suppression of mitotic recombination between repetitive sequences. Cloning and sequencing of this gene showed that it encodes a protein homologous to *Escherichia coli* DNA topoisomerase I, a type I enzyme that shares no significant sequence similarity with eukaryotic DNA topoisomerase I and was until then thought to exist only in prokaryotes. Based on the sequence data, the yeast gene was named *TOP3*, and its product, DNA topoisomerase III (17). Biochemical studies of purified yeast DNA topoisomerase III have confirmed the prediction that the protein, similar to *E. coli* DNA topoisomerase I, catalyzes the removal of negative but not positive supercoils (18). Both *in vitro* and *in vivo* studies suggest that relative to yeast DNA topoisomerases I and II, yeast DNA topoisomerase III has a much weaker activity in the removal of negative supercoils (18). It is therefore surprising that top3$^-$ TOP1$^+$ TOP2$^+$ cells lacking the weak supercoil-removal activity of DNA topoisomerase III but possessing two robust DNA topoisomerases I and II are nevertheless hyper-recombinogenic or hyper-rec and grow 50% more slowly relative to otherwise isogenic strains (17). Sporulation is also known to be completely blocked in *top3*$^-$/*top3*$^-$ diploids (17).

Recently, inactivation of a yeast gene *SGS1* was found to suppress top3$^-$ phenotypes (19). Sequence analysis indicates that SGS1 protein is homologous to *E. coli* RecQ protein, which possesses a DNA helicase activity and is involved in the RecF pathway of recombination (19). TOP3 and SGS1 proteins also appear to physically interact (19), which is reminiscent of the association between a bacterial DNA topoisomerase I-like domain and a helicase-like domain in the enzyme "reverse gyrase," an activity found in hyperthermophiles that positively supercoils DNA (20).

Yeast *TOP3* gene was also found recently (21) to be abutted head-to-head to a gene *EST1*, mutations in the latter lead to progressive shortening of telomeric repeats and cell senescense (22). The initiation codons of the divergent pair of genes are separated by 258 bp, and this close spacing suggests that the two might be coregulated (21). Mutations in *TOP3* itself shorten telomeric repeats as well as destabilize subtelomeric elements (21). Whether these effects are related to *EST1* function or they represent a manifestation of the general hyper-rec phenotype of *top3* mutants is uncertain.

Whereas DNA topoisomerases I and II are known to be ubiquitous and have been studied extensively, no other eukaryotic DNA topoisomerase III was reported since the 1989 finding of the yeast enzyme. Because of the known phenotypes of yeast DNA topoisomerase III and the general relation between genome stability and genetic disorders including cancer, we decided to examine whether multicellular organisms also possessed such an enzyme, and if so, whether the activity might be involved in suppressing mitotic recombination. In this communication, we report the identification of human DNA topoisomerase III.‡

MATERIALS AND METHODS

Cloning and Characterization of Human *TOP3* cDNA. The GenBank expressed sequence tag data base was first searched for reading frames that shared sequence similarities with yeast DNA topoisomerase III, using the Blast algorithm (23). Among the candidates identified, a 224-nt-long entry R45840 (24) had the potential of coding for a polypeptide with a heptameric stretch Gly-Ile-Gly-Thr-Asp-Ala-Thr identical to residues 544 to 550 of the yeast enzyme. The extent of similarity outside this heptameric identity was limited, but

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U43431).

could be improved by the deletion of a nucleotide around the 85th position in the reported sequence to switch the reading frame. To test further whether the R45840 tag might have been derived from human *TOP3* cDNA, oligodeoxynucleotide primers were synthesized based on the R45840 sequence, and used to screen an amplified plasmid library of human hepatoma cDNA (Austral Biologicals, San Ramon, CA) by PCR. A single candidate was identified in the entire pool of about 1 million independent transformants in the original library. Nucleotide sequencing within the cDNA insert identified an open reading frame highly homologous to the C-terminal half of yeast DNA topoisomerase III sequence. The entire 224-bp sequence of R45840 was contained within the hepatoma cDNA clone, but several mismatches were found between the two, including an extra T at position 87 of the 224-bp expressed sequence tag. Radiolabeled DNA derived from the hepatoma cDNA insert was then used to screen a Jurkat T-cell cDNA library in phage λ (Stratagene). About 70 positives among a total of about 1.5 million phage plaques were identified. One clone with a 3.7-kb insert was sequenced in its entirety, and three others were sequenced in parts, by the dideoxynucleoside triphosphate chain termination method.

Determination of the Chromosomal Location of the Gene. DNA samples from a panel of human–rodent somatic cell hybrids, each of which contains one unique human chromosome (Mapping Panel No. 2, Coriell Cell Repositories, Camden, NJ), were used in the identification of the human chromosome containing *TOP3*. Approximately 5 μg of DNA from each of the somatic hybrids were digested with *Eco*RI, and blot hybridization of the restriction fragments resolved by agarose gel electrophoresis was carried out using a ^{32}P-labeled probe obtained by copying cloned human *TOP3* cDNA. *Eco*RI digests of human, mouse, and Chinese hamster genomic DNA were included in this experiment to serve as references.

The chromosomal position of *TOP3* was measured by fluorescence *in situ* hybridization. Several pairs of synthetic oligodeoxynucleotides 20 bp in size were synthesized and tested for suitability as primers for the identification of *TOP3* genomic clones by PCR. Using human genomic DNA as the template, the pair 5′-CACAGTGGAGATCGACATCG-3′ and 5′-TCCATGAGGGCAATGAGGTC-3′ was found to give a clean 377-bp product containing a 155-bp intron. PCR-based screening of the DuPont–Merck Pharmaceutical Company Human Foreskin Fibroblast P1 library #1 (DMPC-HFF#1) was then carried out by Genome Systems (St. Louis), using this pair of primers. Two positives, DMPC-HFF#1–371-G4 and DMPC-HFF#1–401-E11, were found. DNA samples were prepared from these clones, and analysis by PCR using several pairs of primers for amplification of the 5′ and 3′ terminal parts of the coding sequences showed that both genomic clones contained the entire coding sequence of *TOP3*. DNA of DMPC-HFF#1–401-E11 was used as the probe in chromosomal mapping by fluorescence *in situ* hybridization (performed by Genome Systems).

Expression of Human DNA Topoisomerase III in Yeast. A human *TOP3* cDNA segment was used to replace the yeast *TOP2* segment between *Bsa*BI and *Sma* I sites in a plasmid YEpTOP2PGAL1 (25), which was constructed for overexpression of yeast DNA topoisomerase II from the inducible GAL1 promoter. The junction between the 3′ end of the human *TOP3* cDNA and the vector was created by direct ligation of a repaired *Hin*dIII end past the termination codon of human cDNA to the *Sma* I end of the vector. The 5′ terminal region of the human cDNA was joined to the vector sequence through the construction of a fragment, by PCR, from the initiation codon of the cDNA to a downstream *Pml* I site. A linker sequence 5′-GTGGTACCCC-3′ containing a *Kpn* I site was introduced between the *Bsa*BI site of the vector and the human *TOP3* initiation codon during these cloning steps. The sequence of the region introduced by PCR was confirmed by sequencing. The final expression plasmid, YEphTOP3, is expected to express a recombinant human DNA topoisomerase III with 14 extra amino acids added to the N terminus of the wild-type enzyme, 11 of which were derived from the N terminus of yeast DNA topoisomerase II, and the remaining 3 from the linker introduced for cloning convenience. Two derivatives of YEphTOP3 were also made. In YEphtop3–Y571, the human cDNA was derived from a different allele in which codon 571 encodes a tyrosine instead of cysteine. In YEphtop3–Y337F, the Tyr-337 codon for the putative active site tyrosine in YEphTOP3 was replaced by a phenylalanine by site-directed mutagenesis, using a commercial kit (Clontech).

Relaxation of Negatively Supercoiled DNA by Human DNA Topoisomerase III. Each of the three expression plasmids YEphTOP3, YEphtop3–Y571, and YEphtop3–Y337F was used to transform a *S. cerevisiae* strain JEL1 Δ*top1*, which was derived from JEL1 (26) by the procedure described previously (27). Transformed cells were grown at 30°C in a uracil-minus minimal medium supplemented with 2% raffinose, and expression of the human protein was induced by the addition of galactose to 2%. Six hours after the addition of galactose, cells were pelleted from the cultures and extracts were prepared as described (28). Highly negatively supercoiled pBluescript DNA (Stratagene) was prepared by treatment of the DNA with vaccinia virus topoisomerase I in the presence of excess ethidium. After successive phenol and 2-butanol extractions to remove ethidium, the DNA was ethanol-precipitated and resuspended for assay of DNA relaxation activity. Each assay contained 0.5 μg of DNA in 50 μl of 40 mM Hepes·NaOH (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM KCl, 0.1 mM EDTA, and ≈70 μg of total cellular protein. Reactions were terminated by phenol extraction, and the samples were briefly treated with RNase A before analysis of the DNA products by two-dimensional electrophoresis. A fraction of each sample was loaded in a sample well of a 0.9% agarose gel slab. Electrophoresis buffer was 50 mM Tris·borate, 1 mM EDTA, and 3 μg chloroquine diphosphate per ml for the first dimension, and the same buffer plus 0.5 μg ethidium bromide per ml was used for the second dimension. Various DNA species were visualized by blot hybridization.

RESULTS

Human *TOP3* Encodes a Protein Homologous to Members of the *E. coli* DNA Topoisomerase I Subfamily. A consensus translational initiation signal GCCGCCATGG (29) was identified near the 5′ end of the sequence. Starting from the underlined ATG within this initiation signal, the open reading frame encodes a 976-residue protein. An alternative start site could be CTGAGGATGA further upstream, which would add another 25 residues to the N terminus of the encoded protein. A polyadenylylation signal AATAAA (30) was found downstream of the stop codon, and a string of adenosines was found 14 nt further downstream in one of the cDNA clones.

Fig. 1 depicts an alignment of the amino acid sequences of the human protein, yeast DNA topoisomerase III, and *E. coli* DNA topoisomerases I and III. For the region present in all four polypeptides, the human protein resembles yeast DNA topoisomerase III more than the two *E. coli* enzymes (44% identity and 61% sequence similarity for the former pair versus around 24% identity and 44% sequence similarity for the latter pairs). On the basis of results, the human gene containing the cloned cDNA sequences is denoted *TOP3*, and the protein it encodes is denoted DNA topoisomerase III. The aligned sequences suggest that Tyr-337 of the human protein corresponds to Tyr-319 of *E. coli* DNA topoisomerase I, which has been shown to be the active site residue in the breakage and rejoining of DNA (31). In addition to the active site tyrosine, a number of residues that are conserved among all bacterial

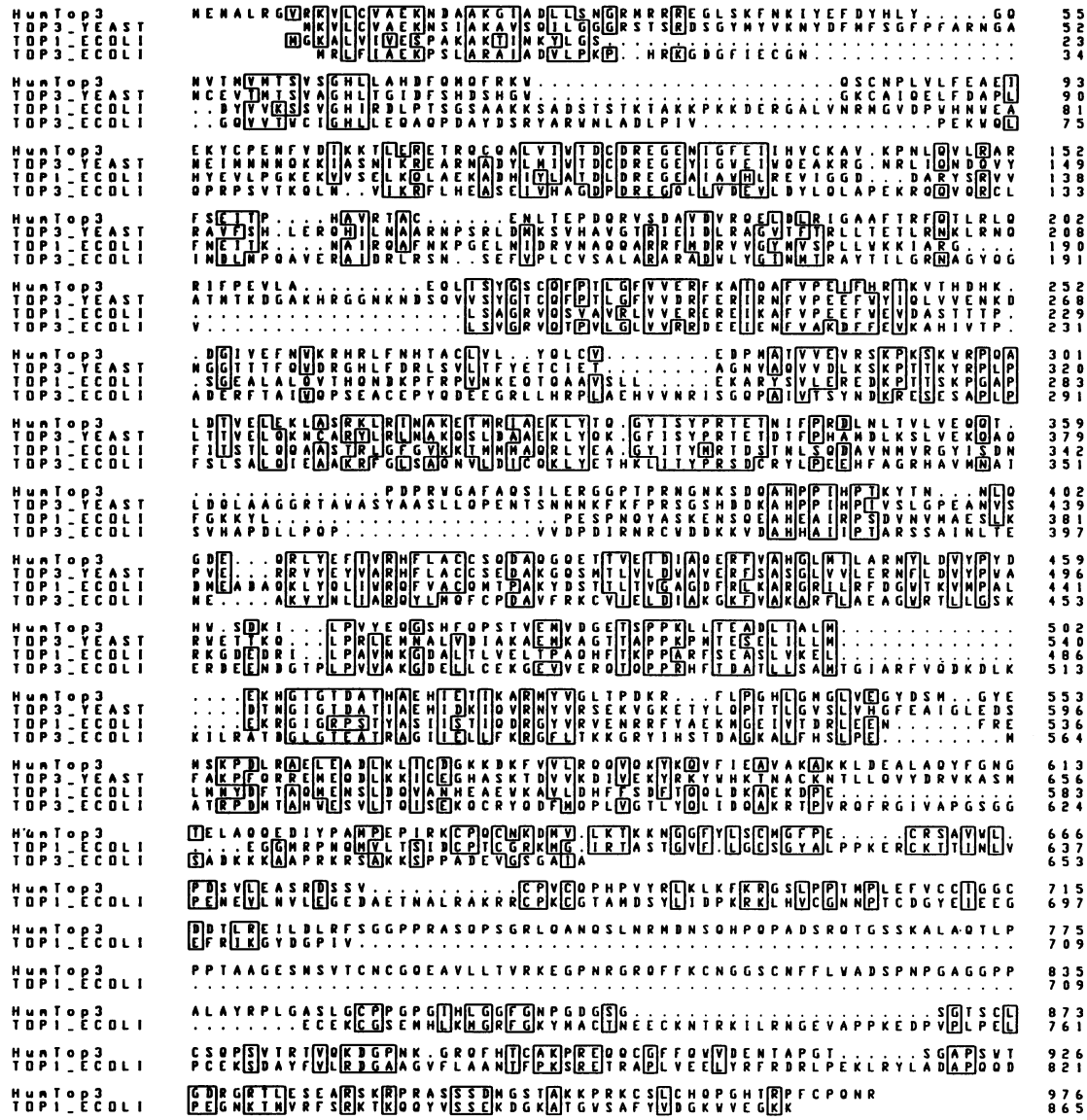

FIG. 1. An alignment of the amino acid sequences of human, *S. cerevisiae*, and *E. coli* DNA topoisomerases I and III.

and yeast topoisomerases of this subfamily are also found at corresponding positions in the human protein (see Fig. 1).

Two differences were present in the nucleotide sequences of five different cDNA clones. For the hepatoma cDNA clone and one of the T-cell clones, codon 571 codes for cysteine (TGT); for three other T-cell clones, codon 571 codes for tyrosine (TAT). The other difference occurred at nucleotide 3354: a guanine was present in the T-cell clone with Cys-571, and a cytosine was present in the other clones. Sequencing of the codon 571 region in two human *TOP3* genomic clones from foreskin fibroblasts (see *Materials and Methods*) showed the presence of a TGT triplet at codon 571. In the sequence deposited in GenBank, a TGT triplet and a cytosine are assigned to codon 571 and nucleotide 3354, respectively.

The Overall Organization of the Human TOP3 Protein Is Similar to That of *E. coli* DNA Topoisomerase I. In terms of the overall organization, the 976-aa human DNA topoisomerase III resembles the 865-aa *E. coli* DNA topoisomerase I more than the 654-aa yeast DNA topoisomerase III. The human enzyme has a C-terminal segment of over 300 aa with no counterpart in the yeast enzyme. This C-terminal segment shares significant sequence homology, however, with the C-terminal portion of *E. coli* DNA topoisomerase I (Fig. 1). The C-terminal segment of *E. coli* DNA topoisomerase I contains three motifs with four cysteines in each, and these "tetracysteine motifs" are followed by a 121-aa C-terminal segment. The tetracysteine motifs have been implicated in the binding of three Zn(II) atoms (32), and at least the first of these is required for the DNA relaxation activity of the *E. coli* enzyme (33). The 121-aa C-terminal domain is dispensable for relaxation activity but appears to bind single- and double-stranded DNA and make the enzyme more processive and less salt-sensitive (34). As shown in Fig. 1, the six cysteines at positions 633, 636, 654, 660, 680, and 683 of the human protein appear to correspond to the N-terminal proximal six cysteines in the tetracysteine motifs of *E. coli* DNA topoisomerase I; the C-terminal extremity of the human protein also appears to be homologous to that of the bacterial enzyme (Fig. 1). Alignment of sequence segments within the human TOP3 protein also reveals the presence of two 28-residue repeats (amino acids 787–814 and 871–898), which might have resulted from a duplication event.

Human TOP3 Protein Expressed in Yeast Can Partially Relax a Highly Negatively Supercoiled DNA. The sequence data strongly suggest that the cloned human cDNA encodes a topoisomerase of the *E. coli* DNA topoisomerase I subfamily. This was confirmed by expressing the human cDNA in yeast and examining for activity that relaxes negatively supercoiled

DNA. Extracts of cells bearing a plasmid expressing human TOP3 with either a cysteine or a tyrosine codon for residue 571 were examined. As a control, extracts of cells expressing a mutant human DNA topoisomerase III Y337F, in which a phenylalanine replaces the putative active site residue Tyr-337, were also assayed. The results are depicted in Fig. 2.

Six samples were analyzed by two-dimensional agarose gel electrophoresis. Lanes 1, 5, and 6 in Fig. 2 show three reference DNA samples. They are untreated highly negatively supercoiled DNA substrate used in the assays, pBluescript DNA before conversion to the highly negatively supercoiled form, and pBluescript DNA relaxed to completion with vaccinia virus topoisomerase in the absence of ethidium, respectively. Lanes 2–4 show the highly negatively supercoiled DNA after treatment with extract of cells bearing YEphtop3-Y337F, YEphTOP3, and YEphtop3-Y571, respectively. A low level of DNA relaxation activity was present in extract of cells harboring YEphtop3–Y337F, as indicated by the spreading of the DNA from the position of the untreated supercoiled form (denoted by S in lane 1) toward the left (lane 2). This low level of activity was most likely due to the presence of yeast DNA topoisomerase III in the extract. Treatment of the DNA with extract of cells bearing YEphTOP3 resulted in a much larger change in the linking numbers of the input DNA (lane 3). Removal of negative supercoils was incomplete, however, and a comparison of the patterns shown in lanes 3 and 5 shows that after treatment with extract of cells bearing YEphTOP3, the DNA remained more negatively supercoiled than native pBluescript. Extract of cells bearing YEphtop3–Y571 (lane 4) contained relaxation activity significantly higher than the background level (lane 2), but lower than extract of cells bearing YEphTOP3 (lane 3).

Human *TOP3* Is a Single-Copy Gene Located on Chromosome 17p11.2–12. To determine the chromosomal location of human *TOP3* gene, *Eco*RI digests of DNA samples of a panel of human–mouse somatic hybrid lines with one different human chromosome in each were resolved by agarose gel electrophoresis. For reference, human, mouse, and Chinese hamster genomic DNA were similarly analyzed. Blot hybridization with a ^{32}P-labeled probe derived from human *TOP3* cDNA revealed that only DNA from the somatic hybrid containing human chromosome 17 displayed a compound pattern of human-derived and mouse-derived radiolabeled bands (data not shown). Thus human *TOP3* is located on chromosome 17.

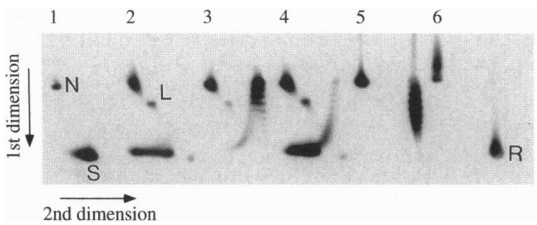

FIG. 2. Assays of extracts of yeast cells that had been induced to express human DNA topoisomerase III. A highly negatively supercoiled DNA prepared from a 3-kb pBluescript DNA was used as the substrate in these assays, and DNA samples were analyzed by two-dimensional agarose gel electrophoresis to resolve the various topological forms of the DNA. Untreated substrate DNA (lane 1) migrated as an unresolved cluster denoted by S; a small amount of nicked (N) DNA was also present in the sample. Under the two-dimensional gel electrophoresis conditions employed, negatively supercoiled pBluescript DNA isolated from *E. coli* cells resolved into a ladder of topoisomers shown in panel 5, and completely relaxed pBluescript DNA showed as a spot (R) at the lower right corner (lane 6). Lanes 2–4 show the two-dimensional gel electrophoretic patterns of the highly negatively supercoiled DNA substrate after treatment with extract of yeast Δ*top1* cells bearing YEphtop3-Y337F, YEphTOP3, or YEphtop3-Y557, respectively, which contained recombinant human DNA topoisomerase III with the active site residue Tyr-337 substituted by a phenylalanine, with no substitution, or with Cys-557 substituted by a tyrosine, respectively. A faint spot at the position of the completely relaxed pBluescript was seen in the samples treated with the cell extracts. This species was most likely derived from the conversion of nicked DNA by adenylylated DNA ligase in the extracts. A small amount of linear DNA (L) was also formed during treatment with cell extracts.

The chromosomal position of human *TOP3* was determined by fluorescence *in situ* hybridization. Metaphase chromosomes of human lymphocytes were probed with digoxigenin-labeled DNA from a human *TOP3* genomic clone in phage P1, and the hybridized signal in propidine-stained chromosomes was detected by fluorescein-tagged anti-digoxigenin antibodies. The *TOP3*-specific probe was found on the short arm of a group E chromosome. Simultaneous hybridization with the *TOP3*-specific probe and a biotin-labeled probe specific for chromosome 17 centromere sequences, followed by staining with fluoresceinated anti-digoxigenin antibodies and Texas Red-tagged avidin, showed that both probes were located on chromosome 17, confirming the assignment based on mapping of the human gene in somatic hybrids. From the position of the human *TOP3* probe in the metaphase chromosomes, the gene was mapped to the boundary between bands 17p11.2 and 17p12.

DISCUSSION

The involvement of yeast DNA topoisomerase III in the suppression of mitotic recombination between repetitive sequences including telomeric sequences (17, 18), and the suppression of the hyper-rec phenotype of *TOP3* mutants by mutations in *SGS1* (19), pose interesting questions on the maintenance of genome stability. All three yeast DNA topoisomerases display a common theme in their suppression of mitotic recombination (reviewed in ref. 35). Inactivation of yeast DNA topoisomerase I greatly increases the frequency of mitotic recombination within the ribosomal DNA gene cluster, but not between other repetitive sequences (36). Destabilization of the ribosomal DNA cluster was also observed when cells with a temperature-sensitive *top2* mutation were grown at a semipermissive temperature (36). Even more striking is that in cells of a Δ*top1 top2ts* yeast strain grown at a permissive temperature, over half of the ribosomal DNA genes are present as excised extrachromosomal rings (37). These rings reintegrate into the chromosomal ribosomal DNA cluster if the cells are induced to express a plasmid-borne *TOP1* or *TOP2* gene (37). Two types of models have been proposed to account for the suppression of mitotic recombination by DNA topoisomerases (35). The hyper-rec phenotype may result from excessive supercoiling of particular regions of intracellular DNA in the absence of a topoisomerase activity. Alternatively, the topoisomerases may participate directly in cellular assemblies that actively separate plectonemic synapses between DNA strands, which could otherwise lead to recombination (35). Experimental data are lacking, however, to distinguish these possibilities.

Human DNA topoisomerase III is probably present in many different cell types. The sequence tag R45840 (24) was derived from an adult human heart cDNA, and the cDNA clones reported in this work were obtained from a hepatoma and a T-cell library. Searching the GenBank expressed sequence tag data base for strings within the full-length human *TOP3* cDNA nucleotide sequence identified two additional entries, T98713 and R08081, which appear to be identical "twins" derived from a fetal spleen/liver library. We are uncertain about the significance of the large difference in the relative abundance of *TOP3* cDNA clones in the hepatoma and T-cell libraries (by 1–2 orders of magnitude). There appear to be two alleles in the T-cell *TOP3* gene, with codon 571 specifying a cysteine in one and a tyrosine in the other. Residue 571 in the human enzyme corresponds to Ala-554 in *E. coli* DNA topoisomerase I (Fig. 1). Although the amino acid residue at this position is not

conserved (Fig. 1; see also ref. 38), the presence of a bulky tyrosyl group may introduce a structural perturbation that lowers enzyme activity (Fig. 2).

The identification of human DNA topoisomerase III in this work suggests that enzymes of the *E. coli* DNA topoisomerase I subfamily are ubiquitous and are thus likely to be found in all living organisms. The biochemical similarity and sequence homology between human and yeast DNA topoisomerase III hint at a functional similarity of the enzymes. In view of the interplay between TOP3 and SGS1 proteins in yeast, it is interesting to note that a human protein that shares sequence similarity with yeast SGS1 protein has recently been linked to Bloom's syndrome, a rare congenital disease with a mutator genotype (39).

We thank Dr. M.-A. Bjornsti (Thomas Jefferson University Medical School) and Drs. T. Scholl and J. Strominger (Harvard University) for human cDNA libraries. This work was supported by National Institutes of Health Grant GM24544.

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