Human Topoisomerase IIα Possesses an Intrinsic Nucleic Acid Specificity for DNA Ligation

USE OF 5’ COVALENTLY ACTIVATED OLIGONUCLEOTIDE SUBSTRATES TO STUDY ENZYME MECHANISM*

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Kenneth D. Bromberg‡§, Chris Hendricks¶, Alex B. Burgin¶¶, and Neil Osheroff***‡‡‡

From the Departments of Biochemistry and Medicine (Hematology/Oncology), Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, the Biology Department, San Diego State University, San Diego, California 92182-4614, and Emerald BioStructures, Bainbridge Island, Washington 98110

Despite the importance of topoisomerase II-mediated DNA ligation to the essential physiological functions of the enzyme, the mechanistic details of this important reaction are poorly understood. Because topoisomerase II normally does not release cleaved DNA molecules prior to ligation, it is not known whether all of the nucleic acid specificity of its cleavage/ligation cycle is embodied in DNA cleavage or whether ligation also contributes specificity to the enzyme. All currently available ligation assays require that topoisomerase II cleave the initial DNA substrate before rejoining can be monitored. Consequently, it has been impossible to examine the specificity of DNA ligation separately from that of scission. To address this issue, a cleavage-independent topoisomerase II DNA ligation assay was developed. This assay utilizes a nicked oligonucleotide whose 5’-phosphate terminus at the nick has been activated by covalent attachment to the tyrosine mimic, p-nitrophenol. Human topoisomerase IIα and enzymes with active-site mutations that abrogated cleavage activity ligated the activated nick by catalyzing the direct attack of the terminal 3’-OH on the activated 5’-phosphate. Results with different DNA sequences indicate that human topoisomerase IIα possesses an intrinsic nucleic acid specificity for ligation that parallels its specificity for DNA cleavage.

Topoisomerase II is an essential enzyme that modulates DNA topology in eukaryotic cells by passing an intact double helix through a transient double-stranded break that it generates in a separate DNA segment (1–7). This double-stranded DNA passage reaction is required for a number of critical nuclear processes including DNA replication, transcription, recombination, and chromosome segregation (1–7).

For topoisomerase II to carry out its catalytic DNA strand passage reaction, it must be able to cleave and ligate the genetic material in a tightly controlled fashion. DNA cleavage, the first of these reaction steps, is required to open the nucleic acid gate that is used for translocation of the second helix (2, 3, 5, 6, 8, 9). During the scission event, topoisomerase II forms covalent bonds between its active site tyrosyl residues and the newly generated 5’-DNA termini (10–12). This covalent intermediate is known as the cleavage complex. The loss of cleavage activity arrests the catalytic cycle of topoisomerase II prior to its DNA strand passage event, thereby blocking all of the catalytic functions of the enzyme (5, 13). Cells can tolerate the partial loss of topoisomerase II-mediated DNA scission (1, 5).

However, if the bulk of the enzyme molecules in a given cell are prevented from cleaving DNA, that cell ultimately will perish from mitotic failure (1, 5, 13).

DNA ligation, the second of these reaction steps, is required to reseal the nucleic acid break generated by cleavage. Ligation is necessary to maintain chromosomal integrity throughout the topoisomerase II catalytic cycle. If this activity is impaired, cellular levels of topoisomerase II-associated DNA breaks rise (2, 5, 14, 15). Because these transient breaks can be converted to permanent double-stranded breaks by the actions of DNA tracking systems, high levels of topoisomerase II-DNA cleavage complexes can initiate recombination events that generate chromosomal aberrations such as insertions, deletions, and translocations (2, 5, 14–17). When the concentration of these permanent DNA breaks overwhelms the cell, programmed death pathways are triggered (15, 18). Thus, the loss of ligation by even a few molecules of topoisomerase II has the potential to instigate a number of deleterious physiological effects.

The potentially lethal nature of topoisomerase II has led to the development of several clinically important anticancer drugs that target the enzyme (2, 5, 15, 19, 20). Many of these drugs increase levels of topoisomerase II-associated DNA breaks specifically by inhibiting the DNA ligation reaction of the enzyme (2, 5, 21–23). Despite the importance of DNA ligation to the physiological and pharmacological functions of topoisomerase II, many critical issues have yet to be addressed. One of the more intriguing questions relates to the nucleic acid specificity of the enzyme. Is the specificity of topoisomerase II embodied entirely in its cleavage step, or does the enzyme also possess an intrinsic nucleic acid specificity for DNA ligation?

It has long been known that topoisomerase II cuts DNA at a unique spectrum of sites. Because the enzyme forms a covalent attachment with its cleaved DNA intermediate and does not release products prior to resealing the nucleic acid break (10–12), topoisomerase II has evolved to ligate only those DNA sequences that it previously has cut. Thus, it is not clear whether the enzyme also maintains a built-in nucleic acid specificity for ligation. In other words, does topoisomerase II simply ligate DNA molecules because they have been cut by its scission reaction, or does it rejoin them because it has a distinct ability to recognize those molecules?

Several experimental approaches have been used to study...
the DNA ligation reaction of topoisomerase II (12, 21, 24–27). However, these approaches all suffer from the same shortcoming: they are restricted by the fact that topoisomerase II must cleave the initial DNA substrate before ligation can be monitored. This requirement has made it impossible to address the specificity of the ligation reaction separately from that of DNA cleavage.

Therefore, to overcome the limitation of existing systems, a cleavage-independent topoisomerase II DNA ligation assay was developed. This system monitors the ability of human topoisomerase IIα to ligate a nicked oligonucleotide whose 5′-phosphate terminus has been activated by covalent attachment to the tyrosine mimic, p-nitrophenol. The results provide strong evidence that the human enzyme possesses an intrinsic nucleic acid specificity for DNA ligation. Furthermore, this ligation specificity parallels the sequence preference for cleavage and appears to be driven by chemical steps rather than the binding affinity of the enzyme for its DNA substrate.

**Experimental Procedures**

**Synthesis of 5′-p-nitrophenyl (5′-pNP)DNA**—The protocol was similar to that described previously for the synthesis of 3′-pN-linked DNA (28). Oligonucleotides were synthesized on a 1-μmol scale, base-deprotected in concentrated NH₄OH at 50 °C for 16 h, and dried in vacuo. Dried pellets were resuspended in 200 μl of 100 mM MES (pH 5.5) and 2 mM MgCl₂, and any insoluble material was removed by centrifugation. Aliquots of 200 μl of 3′-p-nitrophenol (Alrich, Milwaukee, WI) in acetonitrile and 0.048 g of 1-[3-(dimethylamino)propyl]-3-ethycarbodi- imide–HCl (Alrich, Aldrich) were added, and the resulting mixture was shaken at room temperature for 12 h. The aqueous layer was extracted three times with 500 μl of ethyl acetate, and DNA was ethanol-purified from the aqueous precipitate. The 5′-derivatized DNA was purified by union exchange high pressure liquid chromatography using a Dionex DNA-PAC column, 0–1 M NH₄Cl gradient buffer in 50 mM phosphate, pH 7.0. Pure fractions were concentrated in vacuo and stored in H₂O at −20 °C.

**Preparation of Oligonucleotides**—The following oligonucleotides were prepared on an Applied Biosystems DNA synthesizer. 1) A 47-mer corresponding to residues 80–126 of pBR322 and its complementary oligonucleotide. The sequences of the top and bottom strands were 5′-CCGTGTATGAAATCTAACAATG | CGCTATGCTATCCTGCGACCCGGTTAAACGCT-3′ and 5′-ACGGTGCCGAGGATGAACTGACG | AACGCAAGGTTGACAGCGTGTCTGCTCGGTCCGACGC-3′, respectively. Points of DNA cleavage mediated by topoisomerase II in these two sequences are denoted by arrows. 3) A 50-mer corresponding to residues 31–80 of pBR322 and its complementary oligonucleotide. The sequences of the top and bottom strands were 5′-TGGACATGATTATCTACGCG | TCCACGCTCTGCTGAACAAGAAA-3′ and 5′-TCTTGGGTTGTACCCGCCG | TGGCTGCTATGCTACG-3′, respectively. Points of DNA cleavage mediated by topoisomerase II in these two sequences are denoted by arrows. The overlapping (denoted by underline) mutagenic primer 5′-GGCAAGGTTGACAGCGTGTCTGCTCGGTCCGACGC-3′ and the overlapping (denoted by underline) mutagenic primer 5′-GGCAAGGTTGACAGCGTGTCTGCTCGGTCCGACGC-3′ are 5′-GAGAAATTGCTGACCCAGCAGTACCGTACAG-3′ (Tyr → Phe) or 5′-GAGAAATTGCTGACCCAGCAGTACCGTACAG-3′ (Tyr → Ala). Altered codons are indicated in boldface. The mutagenized fragments were reinserted into YEpWOB6, and the sequences of the inserts and DNA junctions were confirmed.

**Purification of Human Topoisomerase IIα—**Wild-type human topoisomerase IIα was expressed and purified from Saccharomyces cerevisiae as described previously (31, 32). Mutant human topoisomerase IIα proteins containing an active site Phe (Y805F) or Ala (Y805A) were purified as above from an isogenic yeast strain carrying the top2-4 temperature-sensitive allele at the top2-4 chromosomal locus (33). The top2-4 enzyme is inactive above 35 °C. Thus, the use of this strain eliminates any possible contaminating DNA cleavage by yeast topoisomerase II in ligation assays that used enzymes with altered active sites.

**DNA Ligation**—DNA ligation reactions contained 200 nM wild-type or mutant human topoisomerase IIα and 10 nM activated nicked oligonucleotide in a total of 20 μl of reaction buffer (10 mM Tris-HCl, pH 7.9, 135 mM KCl, 7.5 mM CaCl₂, 0.1 mM EDTA, and 2.5% glycerol). Ligation was initiated by the addition of topoisomerase IIα. Reaction mixtures were incubated at 37 °C, and ligation was stopped by the addition of 2 μl of 10% SDS followed by 1 μl of 375 mM EDTA, pH 8.0. Samples were digested with protease K, ethanol-precipitated, and resolved by electrophoresis in 7 M urea, 14% polyacrylamide gels. DNA ligation products were visualized and quantified on a PhosphorImager (Amerham Biosciences).

**DNA Cleavage**—DNA cleavage assays were carried out as described previously (29, 32). Reaction mixtures contained 200 nM wild-type human topoisomerase IIα and 10 nM double-stranded oligonucleotide in 20 μl of reaction buffer. The reactions were incubated at 37 °C for 15 min, and cleavage intermediates were trapped by adding 2 μl of 10% SDS followed by 1 μl of 375 mM EDTA, pH 8.0. Samples were treated and analyzed as described above for ligation reactions.

**Topoisomerase IIα DNA Binding**—An electrophoretic mobility shift...
observed in the absence of enzyme (\textsuperscript{58}). The positions of the 5' termini of the 22-mer DNA substrate were visualized and quantified as described above.

Cleavage-independent DNA ligation by human topoisomerase IIa. The central sequence of the activated pBR322 substrate is depicted and labeled as per Fig. 1. Polycrylamide gels of typical DNA ligation reactions catalyzed by wild-type (WT) or active-site Phe (Y805F) human topoisomerase IIa are shown. No ligation was observed in the absence of enzyme (\textsuperscript{58}). The positions of the 5'-end labeled 22-mer (DNA) and expected ligation product 47-mer marker (\textit{M}) are indicated. DNA ligation assays catalyzed by WT (\textbullet{}), Y805F (\textdagger{}), or active-site Ala (Y805A, \textdaggerdbl{}) topoisomerase IIa are quantitated below. Error bars represent the S.D. of three independent experiments.

**RESULTS AND DISCUSSION**

Although the ability to catalyze efficient DNA ligation is critical to the essential functions of topoisomerase II, the mechanistic details of this reaction are poorly understood. Consequently, several different assays have been developed to study the DNA ligation reaction of topoisomerase II. One assay traps a kinetically competent cleavage complex by manipulating the divalent cation, making it possible to monitor a unidirectional religation reaction (12, 21, 24). Others follow ligation by shifting cleavage complexes to sub-optimal salt or temperature conditions (26, 27). A final assay utilizes “suicide substrates” for topoisomerase II that enable a cleaved DNA segment to dissociate from the enzyme and allow the ligation of the internal nick. The terminal 5'-phosphate at the nick is activated by attachment to a \(p\)-nitrophenyl (\(5'\)-\(p\)-NP) moiety, which is designed to mimic the linkage between the active site tyrosyl residue of topoisomerase II (Tyr-805 in human topoisomerase IIa (34)) and the terminal 5'-phosphate of the cleaved DNA. Previously, it has been shown that type IB topoisomerases and tyrosine recombinases are able to ligate similar 3'-activated substrates (28, 35, 36).

The underlying rationale of the above system (Fig. 1) is that the 5'-\(p\)-NP moiety can replace Tyr-805 as the leaving group. This allows topoisomerase II to catalyze a phosphotransfer reaction in which the terminal 3'-OH at the nick attacks the terminal 5'-phosphate and seals the DNA break. By activating the 5'-phosphate with a synthetic group rather than the active-site tyrosine of the enzyme, it should be possible to monitor ligation under conditions that do not require prior cleavage of the DNA by topoisomerase II.

The initial substrate used for the ligation assay was a double-stranded 47-mer (residues 80–126 from pBR322) that contained a strong, well-characterized topoisomerase II-DNA cleavage site (10, 37, 38). The activated nick was placed on the top strand at the bond normally cleaved by the enzyme (\textsuperscript{58}). Topoisomerase II-mediated DNA cleavage generates a 4-base 5'-overhang. The scissile bond on the complementary strand is shown for reference (Fig. 1, \textit{arrow}). DNA ligation was monitored by following the conversion of the radiolabeled 22-mer (which terminates at the 3'-OH of the nick) to the full-length 47-mer.

Results of a typical DNA ligation assay are shown in Fig. 2. In the presence of wild-type human topoisomerase IIa, \(\sim 40\%\) of the activated DNA substrate was ligated over a 48-h time course. No ligation was observed in the absence of enzyme, indicating that strand sealing was being catalyzed by topoisomerase II. To ensure that ligation was completely independent of cleavage, two mutant human enzymes that lacked their active-site tyrosyl residues were generated. These mutant enzymes contained either phenylalanine (Y805F) or an alanine (Y805A) in place of Tyr-805. Neither displayed any ability to cleave pBR322 plasmid DNA or the oligonucleotide substrate (data not shown).

Both mutant enzymes were able to catalyze the ligation reaction (Fig. 2). The Y805F enzyme was \(\sim 2\)-fold more efficient than wild-type topoisomerase II and ligated \(\sim 80\%\) of the activated oligonucleotide substrate over the course of the assay. The mechanistic basis for the superiority of the Tyr \(\rightarrow\) Phe mutant is not known. However, it may be related to the decreased steric bulk or increased hydrophobicity of a phenylalanine compared with a tyrosine. In contrast, despite the small size of alanine, Y805A topoisomerase II was \(\sim 2\)-fold less active than the wild-type enzyme.

To confirm that ligation of the oligonucleotide substrate was dependent on the presence of the 5'-\(p\)-NP moiety, the ability of human topoisomerase IIa to seal the nick when the terminal 5'-phosphate lacked the activating group was determined (Fig. 3). No ligation was observed in the absence of topoisomerase II or in the presence of the wild-type or mutant enzymes. In contrast, the non-activated nick could be sealed by \textit{Escherichia coli} DNA ligase (Fig. 3).

When topoisomerase II cleaves DNA, it creates a break with an enzyme-linked 5'-phosphate and a free 3'-OH (10–12). To assess whether ligation of the activated substrate reflects this critical attribute of the enzyme, the ability of topoisomerase II to ligate a nick containing a 3'-\(p\)-NP moiety and a free 5'-OH was determined. Neither the wild-type nor the Y805F enzyme
ligated this substrate (data not shown; the Y805A enzyme was not tested).

Pathway of DNA Ligation—Topoisomerase II-mediated ligation of the activated substrate may proceed by two distinct reaction pathways (Fig. 4). In the first pathway, the enzyme catalyzes the direct attack of the terminal 3′-OH on the pNP-linked 5′-phosphate without going through a covalent topoisomerase II-DNA intermediate. In the second pathway, Tyr-805 (or in the case of the mutant enzymes, an alternative amino acid) displaces the activating pNP moiety prior to the attack of the 3′-OH. These two pathways can be distinguished because only the latter requires the formation of a covalent enzyme-DNA intermediate.

To determine which ligation pathway is being utilized by human topoisomerase IIα, the activated 25-mer oligonucleotide was labeled on its 3′ terminus with [α-32P]3′-dATP (Fig. 4, left panel) and used to monitor covalent attachment to the enzyme. Covalent attachment shifts the labeled oligonucleotide to the gel origin (Fig. 4, right panel, arrow). A minor amount of covalently linked oligonucleotide (~2%) was observed in the reactions that included wild-type enzyme. Levels peaked at 6 h (shown in the gel) and decreased at longer times. In contrast, no DNA was linked to the Y805F enzyme at any time over a 48-h time course (Fig. 4 and data not shown).

The enzyme-DNA complex detected with wild-type topoisomerase II may represent a requisite intermediate in the ligation reaction or merely scission of a previously ligated product. To differentiate between these two possibilities, the terminal 3′-OH at the nick was replaced with a 2′-OH. Human topoisomerase IIα is unable to cleave a 2′-5′ linkage. Consequently, any covalent enzyme-DNA complex generated with the 2′-OH substrate would have to represent an intermediate formed prior to the ligation of the activated substrate, as opposed to cleavage of a ligated product. As seen in Fig. 4, neither the wild-type nor the Y805F mutant enzyme were able to ligate the activated substrate that contained a 2′-OH at the nick. Furthermore, no covalent topoisomerase II-DNA complexes were observed with either enzyme. Therefore, it is concluded that topoisomerase II catalyzes ligation of the 5′-pNP oligonucleotide by a direct attack of the 3′-OH on the

![Figure 3](http://www.jbc.org/)  
**Fig. 3.** Topoisomerase II mediated-DNA ligation requires an activated DNA substrate. The sequence of the pBR322 substrate is indicated as in Fig. 2 with the exception that the 5′-phosphate was not activated with a pNP moiety. A polyacrylamide gel of a 48-h time point for DNA ligation catalyzed by wild-type (WT), Y805F (Y → F), or Y805A (Y → A) human topoisomerase IIα is shown. A 2-h time point for DNA ligation by *E. coli* DNA ligase (Lig) is shown for reference. The positions of the 5′-radiolabeled 22-mer (DNA) and expected ligation product 47-mer marker (M) are indicated. No ligation occurred in the absence of enzyme (−TII).

![Figure 4](http://www.jbc.org/)  
**Fig. 4.** DNA ligation proceeds without a covalent topoisomerase II-DNA intermediate. Left, DNA ligation may occur by direct attack of the terminal 3′-OH on the activated 5′ terminus (left pathway) or through a covalent enzyme-DNA intermediate (right pathway). The activated pBR322 sequence is shown as indicated in Fig. 1 with the exception that the 3′ terminus of the activated oligonucleotide is labeled (asterisk), which adds one nucleotide (26-mer). Right, a polyacrylamide gel of a 6-h time point for DNA ligation catalyzed by WT or Y805F (Y → F) human topoisomerase IIα. Ligation substrates contained a terminal 3′-OH or 2′-OH at the activated nick. Covalent attachment of DNA to topoisomerase II shifts the radiolabeled 26-mer to the gel origin (arrow). The 47-mer marker (M), which runs one base below the expected ligation product, is shown. No ligation occurred in the absence of enzyme (−TII). As noted in the scheme (left), human topoisomerase IIα is unable to cleave a 2′-5′ linkage.

* S. D. Cline and N. Osheroff, unpublished result.

* Because the 2′-OH substrate cannot be ligated, it is possible that the enzyme forms a fleeting and unstable covalent bond with the 5′-DNA terminus as part of an abortive reaction. However, if this were the case, the pNP-DNA linkage would be hydrolyzed, which would liberate the activating moiety and decrease the size of the labeled DNA. Because no change in the mobility of the labeled substrate was observed (Fig. 4), this possibility was excluded.
activated 5′-phosphate and does not proceed through a covalent enzyme-DNA intermediate.

**Specificity of DNA Ligation**—Despite the importance of topoisomerase II-mediated DNA cleavage and ligation to genomic integrity, the mechanistic details of these reactions remain enigmatic. Type II topoisomerases display a reproducible but poorly understood site specificity for DNA cleavage (10, 11, 39–41). One of the more intriguing aspects of ligation is whether the enzyme also maintains an intrinsic specificity for DNA strand joining. Is all of the nucleic acid specificity in the topoisomerase II cleavage/ligation cycle embodied in the DNA cleavage reaction, or does ligation also contribute specificity to the enzyme? Because topoisomerase II normally rejoins only DNA sequences that are covalently attached to its active-site tyrosines (10, 11, 39–41), the enzyme may require no special recognition of molecules to be ligated. Conversely, topoisomerase II may possess a distinct ability to recognize these molecules, and this recognition may contribute to the efficiency of ligation.

Because DNA scission normally is a prerequisite for ligation, it has been impossible to address this important mechanistic issue. However, using the cleavage-independent ligation assay described above, it is possible to discern between these two alternatives.

First, the ability of Y805F topoisomerase II to ligate three different activated oligonucleotides was determined (Fig. 5, left panel). One was the pBR322 sequence used for all previous experiments, which contained a strong DNA cleavage site (10, 37, 38). Another was a sequence from the MLL oncogene, which contained a weak cleavage site located proximal to a leukemic breakpoint (32, 42). The third was an oligonucleotide that contained no cleavage site (denoted NCS) for topoisomerase II (10, 37). Cleavage of the corresponding intact duplex oligonucleotides by wild-type topoisomerase IIa is depicted at right (Cleavage). Error bars represent the S.D. of three independent experiments.

Second, the terminal 3′-base at the nick of the pBR322 sequence was changed from a G to an A, C, or T, respectively. A corresponding change was made in the complementary strand of each oligonucleotide to maintain base pairing. Levels of cleavage for all three substrates were decreased compared with the parent sequence such that G > A > C > T (Fig. 5, right panel). As above, the rates of DNA ligation reflected changes in scission (left panel). Taken together, these findings strongly suggest that human topoisomerase IIa has an intrinsic speci-
ficiency for DNA ligation that mirrors the specificity of its cleavage reaction.

To address the possibility that the results in Fig. 5 simply reflect the binding specificity of topoisomerase II for different oligonucleotides (as opposed to an intrinsic specificity for ligation), the ability of the Y805F enzyme to bind each of the activated oligonucleotides was assessed by an electrophoretic mobility shift assay (Fig. 6). Topoisomerase II displayed a similar affinity for all of the oligonucleotides examined. Furthermore, ligation assays were carried out at a 20:1 ratio of enzyme:oligonucleotide, a ratio (and concentration) at which all of the oligonucleotides are completely bound by the enzyme. This finding leads to two conclusions. First, the results shown in Fig. 5 reflect the specificity of topoisomerase II-mediated DNA ligation rather than a differential binding affinity for the oligonucleotide substrates. Second, the nucleic acid specificity of human topoisomerase IIα appears to be determined by chemical steps in DNA cleavage and ligation as opposed to initial DNA binding.

To extend the above conclusions, the positional specificity for ligation within the pBR322 cleavage site was investigated. For these experiments, the activated nick in the oligonucleotide was shifted either two or four nucleotides downstream from the point of scission. As seen in Fig. 7, neither the Y805F mutant nor wild-type human topoisomerase IIα was able to ligate these nicks. This is despite the fact that the wild-type enzyme cleaved both activated substrates at the original point of scission. Thus, the specificity of topoisomerase IIα is sufficiently strict that even within a strong cleavage site, the enzyme must interact in the proper register to catalyze ligation.

In conclusion, a cleavage-independent assay was developed and used to study the DNA ligation reaction of topoisomerase II. The results indicate that human topoisomerase IIα possesses an intrinsic nucleic acid specificity for ligation. This specificity appears to be driven by chemical steps rather than initial DNA binding and is maintained throughout the cleavage/ligation cycle of the enzyme. Because the enzyme does not normally release cleaved DNA intermediates (2, 3, 5–7, 10–12), the physiological benefits of this specificity are not known. However, by contributing to the efficiency of DNA rejoining, specificity may play an important role in helping to maintain genomic integrity during the double-stranded DNA passage reaction of topoisomerase II. Finally, the cleavage-independent assay provides a unique platform with which to further dissect the mechanism of topoisomerase II-mediated DNA ligation and the action of anticancer agents that target the enzyme.
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