DNA Ligation Catalyzed by Human Topoisomerase IIα†

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ABSTRACT: The DNA ligation reaction of topoisomerase II is essential for genomic integrity. However, it has been impossible to examine many fundamental aspects of this reaction because ligation assays historically required the enzyme to cleave a DNA substrate before sealing the nucleic acid break. Recently, a cleavage-independent DNA ligation assay was developed for human topoisomerase IIα [Bromberg, K. D., Hendricks, C., Burgin, A. B., and Osheroff, N. (2002) *J. Biol. Chem. 277*, 31201−31206]. This assay overcomes the requirement for DNA cleavage by monitoring the ability of the enzyme to ligate a nicked oligonucleotide in which the 5′-terminal phosphate at the nick has been activated by covalent attachment to the tyrosine mimic, *p*-nitrophenol. The cleavage-independent ligation assay was used to more fully characterize the DNA ligation activity of human topoisomerase IIα. Results suggest that the active site tyrosine contributes little to the catalysis of DNA ligation beyond its primary role as an activating/leaving group. Although arginine 804 (the residue immediately N-terminal to the active site tyrosine) has been proposed to help anchor the 5′-DNA terminus during cleavage, conversion of this residue to alanine had only a modest effect on DNA ligation. Thus, it appears that arginine 804 does not play an essential role in DNA strand joining. In contrast, disruption of base pairing at the 5′-DNA terminus abrogated DNA ligation in the absence of a covalent enzyme−DNA bond. Therefore, it is proposed that base pairing represents a secondary mechanism for aligning the 5′-DNA termini for ligation. Finally, the human enzyme appears to ligate the two scissile bonds of a cleavage site in a nonconcerted fashion.

Many of the nuclear processes that are essential for cellular survival also have the capacity to destabilize the genome. For example, while DNA replication is required for cell duplication and the passage of the genetic material from one generation to the next, misincorporation of bases represents one of the major sources of inheritable mutations (*1*−*3*). In addition, recombination enzymes that repair many forms of DNA damage sometimes induce chromosomal insertions, deletions, or translocations (*4*, *5*).

Among the enzymes that regulate the normal activities of DNA, topoisomerase II is potentially one of the most dangerous to the cell (*6*−*11*). Topoisomerase II is required for proper chromosome structure and segregation and plays important roles in DNA replication and recombination (*12*−*14*). The enzyme resolves DNA knots and tangles and alleviates torsional stress in the genetic material by passing an intact double helix through a transient double-stranded break that it generates in a separate segment of DNA (*8*, *10*−*17*). To maintain the integrity of the genome during the DNA strand passage event and activate the DNA termini for ligation, topoisomerase II forms covalent bonds between its active site tyrosines (one per subunit of the homodimeric enzyme) and the 5′ termini of the opened DNA gate (*18*−*20*). This covalent topoisomerase II-cleaved DNA complex is referred to as the *cleavage complex*.

Under normal conditions, the cleavage complex is a fleeting intermediate in the double-stranded DNA passage reaction of topoisomerase II. Consequently, the steady-state concentration of this complex is low and the transient DNA breaks required for the strand passage activity of the enzyme are tolerated by the cell. However, conditions that significantly increase the lifetime or cellular concentration of the cleavage complex lead to the accumulation of permanent breaks in the genetic material (*7*−*11*, *21*, *22*). These breaks trigger numerous recombinagenic and mutagenic events (*6*, *7*, *9*, *21*, *22*) and when present at sufficient levels, induce cell death pathways (*6*, *9*). This potentially lethal aspect of topoisomerase II has been exploited for the treatment of human cancers (*8*−*11*, *23*, *24*). Approximately half of current chemotherapeutic regimens contain drugs that kill cells by increasing the physiological concentration of topoisomerase II−DNA cleavage complexes (*9*−*11*, *23*−*27*). Conversely, there is considerable evidence linking increased, but sublethal levels, of topoisomerase II-mediated DNA breaks to the generation of specific infant and therapy-related leukemias (*21*, *22*, *28*−*31*).

Clearly, both the DNA cleavage and ligation activities of topoisomerase II are critical to the cell. If DNA cleavage

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is impaired, the enzyme cannot carry out its essential strand passage activity (*8*, *32*). Alternatively, if DNA ligation is impaired, the enzyme has the capacity to fragment the genome (*7*−*11*). Previously, it has been considerably more difficult to study topoisomerase II-mediated ligation than cleavage. Because ligation assays historically required the enzyme to cleave a DNA substrate before sealing the nucleic acid break (*20*, *33*−*36*), it has been impossible to examine many fundamental aspects of the ligation reaction. Recently, however, a cleavage-independent DNA ligation assay was developed for human topoisomerase IIα (*37*). This assay eliminates the requirement for DNA cleavage by monitoring the ability of the enzyme to ligate a nicked oligonucleotide substrate in which the 5′-terminal phosphate at the nick has been activated by covalent attachment to the tyrosine mimic, *p*-nitrophenol (*37*). Using this assay, it was determined that the type II enzyme possesses an intrinsic specificity for ligation (*37*) and that there are important mechanistic differences between anticancer drugs that inhibit enzyme-mediated DNA ligation or enhance DNA cleavage (*38*, *39*).

Because the DNA ligation activity of topoisomerase II is critical to the maintenance of genomic stability, the cleavage-independent ligation assay was used to further characterize this important reaction. Results strongly suggest that base pairing represents a secondary mechanism for aligning the 5′-terminal phosphate for ligation and that the human enzyme ligates the two scissile bonds of a cleavage site in a nonconcerted fashion.

EXPERIMENTAL PROCEDURES

Enzymes. Wild-type human topoisomerase IIα and mutant enzymes containing a phenylalanine (Y805F), alanine (Y805A), cysteine (Y805C), or histidine (Y805H) in place of the active site tyrosine (Y805) or an alanine in place of arginine 804 (R804A) were expressed in *Saccharomyces cerevisiae* and purified as described previously (*37*, *40*, *41*). Human topoisomerase IIβ was expressed in *S. cerevisiae* and purified in an analogous manner.

Preparation of Oligonucleotides. A 47-base oligonucleotide corresponding to residues 80−126 of pBR322 and its complement were prepared on an Applied Biosystems DNA synthesizer. The sequences of the top and bottom strands were 5′-CCGTGTATGAAATCTAACAATG↓CGCTCATCGTCATCCTCGGCACCGT-3′ and 5′-ACGGTGCCGAGGATGACGATG↓AGCGCATTGTTAGATTTCATACACGG-3′, respectively. Points of topoisomerase II-mediated DNA cleavage are denoted by arrows. Oligonucleotides spanning the 5′-terminus to the point of topoisomerase II scission on each strand also were synthesized. Oligonucleotides extending from the point of scission to the 3′-terminus of each strand were synthesized and 5′-activated with *p*-nitrophenol according to the method of Bromberg et al. (*37*). A tetrahydrofuran abasic site analogue phosphoramidite (Glen Research) was utilized to incorporate site-specific abasic sites. Single-stranded oligonucleotides were labeled on their 5′ termini with [^{32}P]phosphate and purified as described (*42*). Equimolar amounts of complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

Site-Directed Mutagenesis. Recombinatorial PCR (*43*) was used to mutate the human TOP2α gene as described previously (*37*). To change the active site tyrosine (Y805) to cysteine or histidine, PCR was carried out using the primer GGACTAGCAGAATCCTTGCCACCATGTAGCCTGGTACC and the overlapping (denoted by underline) mutagenic primers GGCAAGGATTCTGCTAGTCCACGA**TGC**ATCTTTACAATGCTCAGC (Y805C) or GGCAAGGATTCTGCTAGTCCACGA**CAC**ATCTTTACAATGCTCAGC (Y805H). For mutagenesis of Arg804, PCR was performed using the primer GGACTAGCAGAATCCTTGCCACCATGTAGCCTGGTACC and the overlapping (denoted by underline) mutagenic primer GGCAAGGATTCTGCTAGTCCA**GCA**TACATCTTTACAATGCTCAGC (R804A). Altered codons are indicated in boldface. All mutations were confirmed by DNA sequencing.

DNA Cleavage. DNA cleavage assays were carried out as described (*41*, *44*). Unless stated otherwise, reaction mixtures contained 200 nM wild-type human topoisomerase IIα and 10 nM double-stranded oligonucleotide in 20 μL of cleavage buffer (10 mM Tris-HCl, pH 7.9, 135 mM KCl, 7.5 mM $CaCl_2$, 0.1 mM EDTA, and 2.5% glycerol). Reactions were incubated at 37 °C for 15 min. Cleavage intermediates were trapped by adding 2 μL of 10% SDS followed by 1 μL of 375 mM EDTA, pH 8.0. Samples were digested with proteinase K and precipitated in ethanol. Reaction products were resolved by electrophoresis in 7 M urea, 14% polyacrylamide gels in 100 mM Tris-borate, pH 8.3, and 2 mM EDTA. DNA cleavage products were visualized and quantified on a Bio-Rad FX Molecular Imager.

Cleavage-Independent DNA Ligation. Cleavage-independent DNA ligation reactions were carried out according to the method of Bromberg et al. (*37*). Unless stated otherwise, assays contained 200 nM wild-type or mutated human type II topoisomerases and 10 nM activated nicked oligonucleotide in a total of 20 μL of 10 mM Tris-HCl, pH 7.9, 135 mM KCl, 7.5 mM $CaCl_2$, 0.1 mM EDTA, and 2.5% glycerol. 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 treated, resolved in denaturing polyacrylamide gels, and analyzed as described above for DNA cleavage reactions.

Cleavage-Dependent DNA Religation. Cleavage-dependent DNA religation assays were carried out as described previously (*44*). Reaction mixtures contained 135 nM wild-type or R804A topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer. DNA cleavage/religation equilibria were established at 37 °C for 6 min. Religation was initiated by shifting reaction mixtures from 37 to 0 °C, and reactions were stopped at time points up to 30 s by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM NaEDTA, pH 8.0. Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reactions were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μL of 60% sucrose in 10 mM Tris-HCl, pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF, heated for 3 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris−acetate, pH 8.3, and 2 mM EDTA that contained 0.5 μg/mL ethidium bromide. DNA religation was monitored by the loss of linear plasmid molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

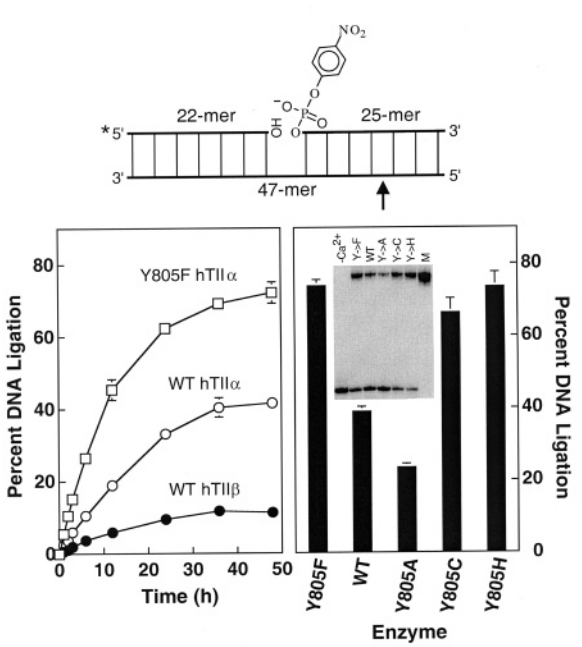

FIGURE 1: Cleavage-independent DNA ligation catalyzed by human topoisomerase IIα. A schematic of the activated DNA substrate (residues 80−126 of pBR322) utilized for DNA ligation of the top strand is shown (*37*). The duplex oligonucleotide contains a nick at the point of scission of a strong topoisomerase II cleavage site (*19*, *65*, *66*). The 5′-terminal phosphate at the nick is activated for DNA ligation by the covalent attachment of *p*-nitrophenol (*37*). The asterisk denotes the position of the 5′-radiolabel, and the arrow indicates the point of topoisomerase II-mediated DNA cleavage on the bottom strand. The left panel shows a time course for DNA ligation catalyzed by wild-type human topoisomerase IIα (WT hTIIα; ○), an active site Phe mutant enzyme (Y805F hTIIα; □), or human topoisomerase II*β* (WT hTII*β*; ●). The right panel shows a 48-h time point for DNA ligation catalyzed by WT, Y805F, or mutant human topoisomerase IIα enzymes in which the active site Tyr has been changed to an Ala (Y805A), Cys (Y805C), or His (Y805H). The polyacrylamide gel in the inset shows 48-h time points for DNA ligation catalyzed by the different topoisomerase IIα enzymes. No ligation was observed in the absence of a divalent cation ($-Ca^{2+}$), and the position of the expected ligation product 47-mer marker (M) is shown for reference. Error bars represent the standard deviations for three independent experiments.

RESULTS AND DISCUSSION

Cleavage-Independent DNA Ligation Catalyzed by Human Topoisomerase IIα. The DNA cleavage and ligation reactions of topoisomerase II are required for the enzyme to carry out its essential cellular functions (*8*, *12*, *14*, *16*, *17*). However, conditions that increase the rate of DNA cleavage or impair the ability of topoisomerase II to ligate nucleic acids can lead to the fragmentation of the genome (*7*−*11*).

Attempts to study the topoisomerase II DNA ligation reaction have been hampered by the inability to isolate strand sealing from the strand scission event. Recently, however, an assay was developed that allows topoisomerase II-mediated DNA ligation to be examined in the absence of cleavage (*37*). This assay is based on the finding that human topoisomerase IIα can ligate a DNA nick whose 5′-terminal phosphate has been activated by attachment to *p*-nitrophenol, which mimics the covalent bond between the cleaved DNA substrate and the active site tyrosine (see Figure 1, top) (*37*, *45*). The ligation reaction occurs by the direct attack of the 3′-OH at the nick on the activated 5′-phosphate within the active site of topoisomerase IIα (no reaction is seen in the absence of enzyme) and does not proceed via a covalent enzyme−DNA intermediate (*37*).

As shown previously, the active site tyrosine of topoisomerase IIα is not required for the enzyme to ligate the activated oligonucleotide substrate (*37*). In fact, a mutant enzyme that contained an active site phenylalanine (Y805F) displayed ligation rates that were ∼2-fold higher than that of the wild-type enzyme (Figure 1), even though it was incapable of cleaving DNA (*37*). An active site alanine enzyme (Y805A) also supported ligation, albeit to a lesser extent than the wild-type enzyme (Figure 1, right panel) (*37*).

The present study extended these findings by examining the ability of topoisomerase IIα that contained an active site cysteine (Y805C) or histidine (Y805H) to ligate the activated substrate. Although these enzymes did not cleave DNA under conditions of the assay (not shown), both ligated the activated substrate at rates that were similar to that of the Y805F enzyme (Figure 1, right panel). These data suggest that neither the aromaticity nor the potential charge of the residue at position 805 is essential to the environment of the active site of topoisomerase IIα for DNA ligation. Rather, the role of the active site tyrosine is primarily one of a nucleophile that attacks the double helix and covalently attaches to the 5′-terminal phosphate of the cleaved DNA.

A potential caveat to this conclusion is the fact that the rate-determining step of the DNA ligation assay has not been determined. The observed rate may reflect the actual chemical step of ligation or a required conformational change in topoisomerase IIα that precedes ligation. However, if the 5′-DNA terminus is activated for ligation by attachment to a *p*-nitrophenyl group instead of the active site tyrosine, it is clear that the nature of this amino acid residue 805 does not dramatically alter the overall process of DNA ligation.

Human cells contain two isoforms of topoisomerase II, α and *β* (*46*−*48*). Topoisomerase IIα is critical for chromosome segregation during mitosis, and levels of the enzyme rise dramatically during periods of cell proliferation (*49*−*51*). In contrast, topoisomerase II*β* appears to be present in most cell types, irrespective of proliferation status, and enzyme levels remain relatively constant over cell and growth cycles (*48*, *50*−*52*). Although topoisomerase II*β* is dispensable at the cellular level, the enzyme is required for neural development (*53*, *54*). Therefore, the ability of wild-type topoisomerase II*β* to ligate the activated oligonucleotide was determined. As seen in Figure 1 (left panel), the *β* isoform was able to seal the activated nick. However, the enzyme was ∼3-fold less efficient than wild-type topoisomerase IIα. The basis for this decreased efficiency is not known, but it probably reflects the ability of the active site of the enzyme to accommodate the added bulk of the *p*-nitrophenyl moiety of the substrate rather than a fundamental difference in mechanism.

Ionic Requirements for Ligation of the Activated DNA Substrate. Ligation of the activated DNA substrate required a divalent cation (Figure 1, inset). Although the assay was supported by several different divalent cations, including Mg^{2+}, Ca^{2+} was chosen for two reasons. First, all known eukaryotic type II topoisomerases generate higher levels of DNA cleavage in the presence of Ca^{2+} than in Mg^{2+} (*33*, *55*). Aside from this increased cleavage, the major properties of DNA scission, including reversibility, salt requirements, and nucleotide specificity, are retained in Ca^{2+}-containing

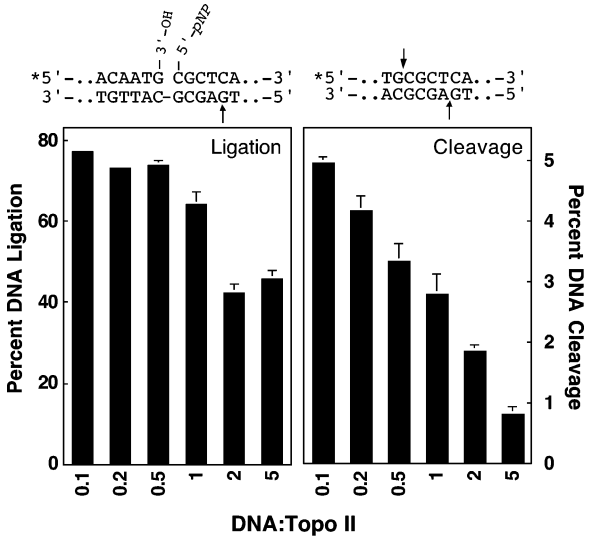

FIGURE 2: Human topoisomerase IIα can recycle during cleavage-independent DNA ligation. The central sequence of the oligonucleotide substrate used for DNA ligation or cleavage is shown above the respective panel. The asterisks denote the positions of the 5′-radiolabel in the substrates and the arrows indicate points of topoisomerase II-mediated DNA cleavage. DNA ligation catalyzed by Y805F human topoisomerase IIα for 48 h at the indicated oligonucleotide:enzyme ratios is quantified in the left panel. DNA cleavage mediated by wild-type human topoisomerase IIα for 15 min at the indicated oligonucleotide:enzyme ratios is quantified in the right panel. Error bars represent the standard deviations for three independent experiments.

reactions (*33*, *55*). Second, a low level of exonuclease activity sometimes was observed in Mg^{2+}-containing reactions over the long time course of ligation. However, most nucleases are inhibited by Ca^{2+} (*56*, *57*), and no degradation of the DNA substrate or product was observed when Ca^{2+} was utilized.

DNA ligation catalyzed by human topoisomerase IIα was supported by a broad range of Ca^{2+} concentration. Optimal levels of ligation were observed at 7.5 mM divalent cation. This optimal Ca^{2+} concentration is similar to that observed in DNA cleavage reactions (data not shown).

High levels of DNA ligation were observed at salt concentrations up to ~185 mM (35 mM KCl is contributed by the enzyme storage buffer), with optimal levels being seen at 135 mM KCl (not shown).

Catalytic Turnover of Topoisomerase IIα in DNA Ligation Assays. To ensure that the cleavage-independent assay measures rates associated with DNA ligation rather than on- or off-rates for enzyme−DNA binding, ligation of the activated oligonucleotide was always monitored under conditions of excess enzyme over DNA (*37*). However, as topoisomerase II does not form a covalent bond with the activated substrate, the enzyme should retain the ability to recycle during the course of the assay (i.e., each molecule of topoisomerase IIα should be able to ligate more than one activated oligonucleotide).

To determine if this was the case, a dilution experiment was carried out (Figure 2, left panel). Ligation remained relatively constant down to a 1:1 oligonucleotide/enzyme ratio. Furthermore, the percent DNA ligation never fell below 40%, even at a 5-fold molar excess of DNA. Because the theoretical maximum level of ligation that would be expected at this substrate:enzyme stoichiometry would be 20%, these data suggest that topoisomerase IIα recycles during the assay.

This finding is in contrast to the stoichiometric DNA cleavage reaction of the enzyme (Figure 2, right panel). Levels of cleavage dropped linearly with enzyme dilution and reflected levels of topoisomerase II−DNA binding (*37*).

Positioning the 5′-DNA Termini for Ligation Catalyzed by Human Topoisomerase IIα. The ability of topoisomerase II to align the DNA termini generated by its cleavage reaction is critical for efficient resealing of the nucleic acid break (*10*, *42*, *58*). The primary mechanism used by the enzyme to align the 5′-DNA termini appears to be the covalent attachment of the 5′-terminal phosphates to the active site tyrosines, rather than base pairing within the cleavage overhang (*58*). In addition, it has been proposed that non-covalent interactions between the DNA and the arginine residue immediately N-terminal to the active site tyrosine play a role in anchoring the 5′ termini of the cleaved DNA (*59*).

Because the cleavage-independent topoisomerase IIα DNA ligation assay does not proceed through a covalent enzyme−DNA intermediate (*37*), it provides a unique opportunity to assess the potential roles of non-covalent enzyme−DNA interactions and base pairing in positioning the 5′ termini. In the absence of the covalent attachment, the contributions of these other interactions should be exacerbated considerably.

First, the role of the arginine 804, which is immediately adjacent to the active site tyrosine in human topoisomerase IIα, was examined. On the basis of structural and modeling studies, Liu et al. (*59*) proposed that arginine 781 of *S. cerevisiae* topoisomerase II (which is equivalent to arginine 804 in the human enzyme) interacts with the 5′-DNA termini during cleavage. The importance of arginine 781 to the catalytic activity of yeast topoisomerase II was confirmed by mutagenesis of this residue to an alanine (*59*). The R781A yeast enzyme failed to complement temperature-sensitive strains deficient in topoisomerase II activity. In addition, the DNA relaxation and cleavage activities of the purified mutant enzyme were ~50- and 10-fold lower than that of wild-type topoisomerase II, respectively.

The equivalent mutation in human topoisomerase IIα (R804A) was generated subsequently by Okada et al. (*60*). This mutant human enzyme failed to complement a temperature-sensitive yeast strain (i.e., *top2-1*) deficient in topoisomerase II activity when expressed from a plasmid under the control of the *GAL1* promoter. On the basis of this result, the authors concluded that arginine 804 was critical to the activity of human topoisomerase IIα and suggested that it was part of the active site of the DNA breakage−reunion reaction (*60*). It should be noted, however, that the authors neither determined the cellular levels of the mutant human enzyme in their study nor purified the enzyme to confirm the effects of the R804 mutation on the activity of topoisomerase IIα. Therefore, we expressed R804A human topoisomerase IIα in yeast, purified the enzyme, and examined its catalytic activity.

The overall catalytic activity (monitored by DNA catenation assays; not shown) and equilibrium levels of DNA cleavage (Figure 3, right panel inset) observed for the mutant enzyme were ~10- and ~5-fold lower, respectively, than those determined for wild-type human topoisomerase IIα. In contrast, the effect of the R804A mutation on cleavage-independent DNA ligation was less pronounced. As seen in

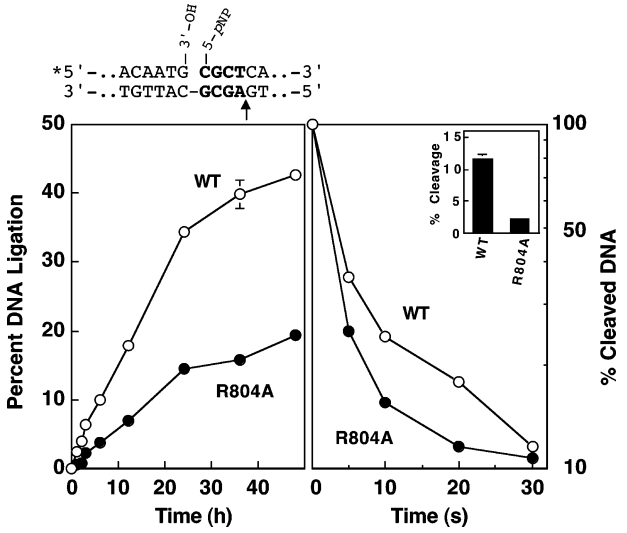

FIGURE 3: DNA ligation catalyzed by R804A human topoisomerase IIα. A time course for cleavage-independent DNA ligation catalyzed by wild-type (WT, ○) or R804A (●) human topoisomerase IIα is shown in the left panel. Error bars represent the standard deviations for two or three independent experiments. The central sequence of the oligonucleotide substrate used for DNA ligation of the top strand is depicted above. The asterisk denotes the position of the 5′-radiolabel, and the arrow indicates the point of topoisomerase II-mediated DNA cleavage on the bottom strand. A representative time course for cleavage-dependent DNA religation catalyzed by wild-type or R804A human topoisomerase IIα is shown in the right panel. The inset in this panel depicts equilibrium levels of DNA cleavage mediated by the two enzymes.

Figure 3 (left panel), the rate of DNA ligation catalyzed by the mutant enzyme was only 2−2.5-fold lower than that of the wild-type enzyme.

Because cleavage-independent DNA ligation does not proceed via a covalent enzyme−DNA intermediate, it is possible that this reaction pathway utilizes different amino acids in the active site of topoisomerase II than does the enzyme-linked reaction. We believe that this is unlikely, because cleavage-independent ligation mirrors many aspects of topoisomerase II-mediated DNA cleavage/religation. For example, DNA sequences that are stronger cleavage sites ligate more quickly in the assay (*37*). In addition, the effects of anticancer drugs in the cleavage-independent assay are similar to those observed in native cleavage/religation reactions, including the mechanism of drug action and the correlation between the nucleic acid specificity of drug-induced cleavage and rates of DNA ligation (*38*, *39*).

However, to confirm that the modest effect of the R804A mutation on cleavage-independent DNA ligation reflects the physiological role of this amino acid residue rather than a fundamental change in the pathway used by topoisomerase IIα to ligate activated nucleic acid substrates, a cleavage-dependent religation assay was employed. In the latter assay, topoisomerase II cuts DNA and becomes covalently attached to the 5′ termini through its active site tyrosine residues. After a DNA cleavage/religation equilibrium is established at 37 °C, religation rates are monitored by decreasing reaction temperatures to 4 °C. At this suboptimal temperature, the enzyme will religate DNA molecules that it already has cleaved but will not reinitiate new rounds of scission. As seen in Figure 3 (right panel), the R804A mutation had no adverse effect on rates of religation when the cleaved DNA was covalently linked to topoisomerase II. If anything, the mutant enzyme religated molecules more quickly than did wild-type topoisomerase IIα. This finding is consistent with the prediction that disruption of non-covalent enzyme−DNA interactions that affect DNA ligation would be masked (to some extent) in the presence of a covalent enzyme−DNA linkage.

The above results suggest that arginine 804 is important to the overall catalytic activity of human topoisomerase IIα. However, while they are consistent with this residue playing a minor role in positioning the 5′-DNA termini, they argue strongly against a critical role for arginine 804 in the DNA ligation event mediated by the human type II enzyme.

It is not known why R804A topoisomerase IIα failed to complement a yeast strain that was deficient for topoisomerase II activity. It may be that the low catalytic activity of the R804A human enzyme was insufficient to support the required cellular functions of topoisomerase II. However, it is notable that the mutant enzyme expressed poorly (expression was <10% of that observed with the wild-type human enzyme) in our system. Thus, it is possible that the failure of R804A topoisomerase IIα to complement deficient yeast resulted from the low levels of enzyme rather than a decrease in catalytic activity.

Second, the role of base pairing in aligning the 5′-DNA termini for ligation was assessed. An earlier study established that in the presence of a covalent attachment between the 5′-DNA termini and the active site tyrosines, disruption of base pairing within the cleavage overhang by the introduction of abasic sites had little effect on rates of ligation catalyzed by human topoisomerase IIα (*58*). Introduction of one or two abasic sites at any of the positions within the 4-base cleavage overhang had no effect on rates of resealing. Even disruption of all 4 base pairs within the cleavage overhang decreased rates of nucleic acid resealing no more than 3-fold.

In the absence of a covalent enzyme−DNA bond, base pairing of the 5′-DNA termini appears to be critical for ligation. As seen in Figure 4, loss of this terminal base pair (by the insertion of an abasic site at the +4-position on the opposite strand) decreased rates of ligation ∼80-fold. Conversely, introduction of an abasic site at either the +3- or +2-position of the opposite strand had a modest effect on rates of ligation, and placement of an abasic site at the +1-position had no effect. Similar results were observed when ligation was monitored on either the top or bottom strand of the activated oligonucleotide substrate (Figure 4). The difference in ligation rates was not due to an altered affinity of topoisomerase II for these substrates. As determined by electrophoretic mobility shift assays, the enzyme displayed a similar binding affinity for the intact oligonucleotide and substrates that contained an abasic site at either the +1- or +4-position (not shown).

Because the loss of base pairing at the 5′-DNA termini ablates ligation, it is possible that a tighter base pair might enhance rates of ligation. To this point, the top strand (which has a C−G base pair at the activated 5′-DNA terminus; see Figure 4) ligates more rapidly than the bottom strand (which has an A−T base pair). (The implication of this rate difference is discussed in the following section.) Therefore, the rate of ligation of the bottom strand was determined when the 5′-terminal A−T base pair was changed to the tighter C−G base pair (Figure 5). Both wild-type human topoisomerase IIα and the Y805F mutant enzyme ligated the

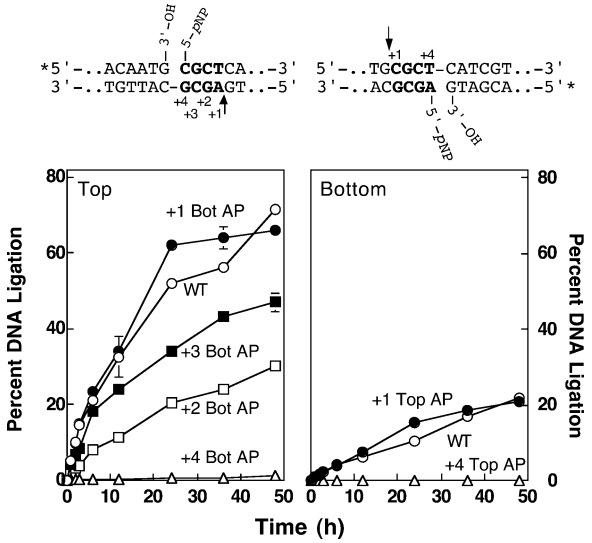

FIGURE 4: Base pairing as a secondary mechanism for positioning the 5′-DNA termini for topoisomerase II-catalyzed DNA ligation. The central sequence of the oligonucleotide substrate used for DNA ligation of the top or bottom strand is shown above the respective panel. The asterisks denote the positions of the 5′-radiolabel in the substrates, and the arrows indicate points of topoisomerase II-mediated DNA cleavage. The numbers on the nonactivated strand indicate the positions where single apurinic/apyrimidinic (AP) sites were inserted into the DNA substrate. The left panel shows a time course of DNA ligation of the top strand catalyzed by Y805F human topoisomerase IIα. The activated DNA sequences used were the wild-type pBR322 substrate (WT, ○) or the substrate in which the +1 (+1 Bot AP, ●), +2 (+2 Bot AP, □), +3 (+3 Bot AP, ■), or +4 base (+4 Bot AP, △) of the bottom strand (relative to the topoisomerase II point of scission) contained an AP site. The right panel shows a time course of DNA ligation of the bottom strand catalyzed by Y805F human topoisomerase IIα. The activated DNA sequences used were the wild-type pBR322 substrate (WT, ○) or the substrate in which the +1 (+1 Top AP, ●) or +4 base (+4 Top AP, △) of the top strand contained an AP site. Error bars represent the standard deviations for two or three independent experiments.

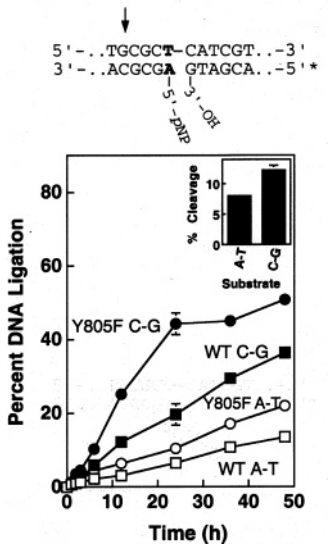

FIGURE 5: Effect of the 5′-DNA terminal base pair of the bottom strand on ligation of the bottom strand. The oligonucleotide substrate used to monitor DNA ligation on the bottom strand is shown. The asterisk denotes the position of the 5′-radiolabel, and the arrow indicates the point of topoisomerase II-mediated DNA cleavage on the top strand. A time course of DNA ligation catalyzed by wild-type (WT, squares) or Y805F (circles) human topoisomerase IIα was monitored on the bottom strand when the +1-position relative to the topoisomerase II point of scission on the bottom strand (in boldface) contained an A−T (○, □) or a C−G (●, ■) base pair. The inset shows DNA cleavage of the bottom strand mediated by wild-type human topoisomerase IIα of the corresponding intact duplex oligonucleotides. Error bars indicate the standard deviations for two to three independent experiments.

bottom strand ∼2.5-fold more rapdily when the 5′-terminal A−T was converted to a C−G. It is possible that the increased rate of ligation for the C−G substrate results from an enhanced recognition of the site by topoisomerase II (note that there is a 1.5-fold increase in equilibrium levels of cleavage as compared to the A−T sequence). However, these data are consistent with a link between base pairing at the 5′ termini and rates of DNA ligation.

Taken together, these findings suggest that base pairing of the 5′-DNA termini acts as a secondary mechanism for aligning the ends of DNA for ligation by human topoisomerase IIα, even in the presence of a covalent enzyme−DNA bond.

Human Topoisomerase IIα Ligates the Two Strands of the Double Helix in a Nonconcerted Fashion. In the presence of the anticancer drug etoposide, there appears to be little communication between the two protomer active sites of human topoisomerase IIα (*38*). This conclusion is based on the finding that inhibition of ligation at one scissile bond by the drug had no effect on the rate of ligation at the other scissile bond.

Additionally, other studies have demonstrated that equilibrium levels of topoisomerase II-mediated DNA breaks at the scissile bonds on the two strands of a cleavage site are not always the same (*18*−*20*, *61*, *62*). Furthermore, at low pH, topoisomerase II generates predominantly single-stranded breaks in both plasmid and oligonucleotide substrates (*20*). These results imply that the type II enzyme may cleave or ligate the two scissile bonds of a cleavage site in a manner that is not totally concerted. However, the coordination of enzyme-mediated DNA ligation has never been addressed directly in the absence of anticancer drugs. Therefore, the cleavage-independent DNA ligation assay was used to examine this issue.

Three lines of evidence indicate that human topoisomerase IIα does not ligate the two strands of the double helix in a concerted fashion. First, the enzyme ligated the two strands of the pBR322 substrate at different rates (Figure 4). The rate of ligation of the top strand was ∼3-fold higher than that of the bottom strand.

Second, disruption of the base pair at the 5′-DNA terminus of one scissile bond (by the introduction of an abasic site), which nearly abrogated ligation on that strand, had no effect on the rate of ligation of the scissile bond on the opposite strand (Figure 4).

Third, conversion of the 5′-terminal base pair on the bottom strand to a C−G, which increased the rate of ligation of the bottom strand ∼2.5-fold, had no effect on the rate of ligation of the top strand (Figure 6).

Conclusions. The cleavage-independent ligation assay was employed to further characterize the DNA ligation reaction catalyzed by human topoisomerase IIα. Results suggest that arginine 804 is important for overall catalytic activity, but does not play an essential role in positioning the 5′-DNA termini for ligation. In contrast, we propose that base pairing

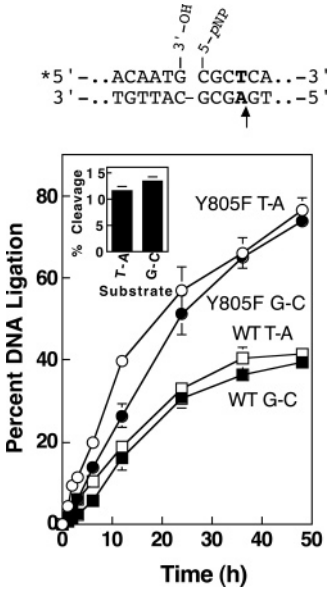

FIGURE 6: Effect of the 5′-DNA terminal base pair of the bottom strand on ligation of the top strand. The DNA substrate used to monitor DNA ligation on the top strand is shown. The asterisk denotes the position of the 5′-radiolabel, and the arrow indicates the point of topoisomerase II-mediated DNA cleavage on the bottom strand. A time course of DNA ligation catalyzed by wild-type (WT, squares) or Y805F (circles) human topoisomerase IIα was monitored on the top strand when the +1-position relative to the topoisomerase II point of scission on the bottom strand (in boldface) contained an A−T (○, □) or a C−G (●, ■) base pair. The inset shows DNA cleavage of the top strand mediated by wild-type human topoisomerase IIα of the corresponding intact duplex oligonucleotides. Error bars indicate the standard deviations for two to three independent experiments.

is a secondary mechanism for aligning the 5′-phosphates for ligation.

Finally, the human enzyme appears to ligate the two scissile bonds of a cleavage site in a nonconcerted manner. It is not clear how (or whether) the apparent lack of coordination between the two protomer active sites of topoisomerase II benefits the catalytic functions of the enzyme. It may simply reflect the residual imprint of an ancestral nicking−closing mechanism shared between type IA and type IIA enzymes (*63*, *64*). Alternatively, this lack of coordination may help to preserve genomic integrity by decreasing the likelihood of a topoisomerase II-generated double-stranded break in the genome prior to the strand passage event.

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