Intrinsic Intermolecular DNA Ligation Activity of Eukaryotic Topoisomerase II

POTENTIAL ROLES IN RECOMBINATION*

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**Drosophila melanogaster** topoisomerase II is capable of joining pieces of DNA that it has cleaved in a trans-cleavage/religation reaction (Gale, K. C. and Osheroff, N. 1990 *Biochemistry* 29, 9538–9545). In order to investigate potential mechanisms for topoisomerase II-mediated DNA recombination, this intrinsic enzyme activity was further characterized. Intermolecular DNA ligation proceeded in a time-dependent fashion and was concentration-dependent with respect to oligonucleotide. The covalent linkage between the enzyme and oligonucleotide of acceptor molecules was confirmed by Southern analysis and alkaline gel electrophoresis. Topoisomerase II-mediated intermolecular DNA ligation required the oligonucleotide to contain a 3'-OH terminus. Moreover, the reaction was dependent on the presence of a divalent cation, was inhibited by salt, and was not affected by the presence of ATP. The enzyme was capable of ligation of a 3' overhang, 3'-overhang, or blunt ends. Single-stranded, nicked, or gapped oligonucleotides also could be used as acceptor molecules. These results demonstrate that the type II enzyme has an intrinsic ability to mediate illegitimate DNA recombination in vitro and suggests possible roles for topoisomerase II in nucleic acid recombination in vivo.

The topological state of nucleic acids is regulated in vitro by enzymes known as type I and type II topoisomerases (1–4). These enzymes influence virtually every aspect of DNA metabolism in eukaryotic cells (5). In addition to roles in chromosome segregation (5–10), DNA replication (11–13), and transcription (14, 15), several lines of evidence indicate that topoisomerases are involved in the process of DNA recombination (3, 4, 10, 16–24). To this point, expression of vaccinia virus topoisomerase I in *Escherichia coli* cells (25) or introduction of exogenous mammalian topoisomerase II into mouse cells (26) greatly increases the frequency of recombination in these systems.

Topoisomerases have been ascribed a number of different roles in the process of DNA recombination. Both topoisomerases I and II are important for the regulation of recombination within the rDNA cluster in yeast (18, 20, 23). Furthermore, topoisomerase II is required for the resolution of chromosomes that have undergone meiotic recombination (10). In addition, evidence suggests that topoisomerases I and II can mediate the DNA cleavage/ligation event that leads to some forms of illegitimate recombination in eukaryotes. For example, sites of *in vivo* recombination are tightly associated with topoisomerase I or II recognition/cleavage sequences (16, 21). Moreover, topoisomerase I- or II-targeted antineoplastic drugs (that stabilize covalent enzyme-cleaved DNA complexes (27, 28)) promote chromosomal translocations and mutations, as well as sister chromatid exchange in treated cells and human patients (29–37). Finally, both enzymes have been shown to carry out illegitimate recombination (16, 17, 19, 24) or intermolecular DNA ligation (38–40) in vitro.

Proposed models for topoisomerase I-mediated illegitimate recombination all rely on the enzyme’s ability to introduce transient single-stranded breaks in the DNA backbone (16, 22, 24). In these models, a single molecule of the type I enzyme joins cleaved nucleic acids to the free termini of separate DNA acceptor molecules by an intermolecular ligation event. In contrast, the only models for topoisomerase II that have been proposed require two separate enzyme homodimers to introduce double-stranded breaks in DNA. In these latter models, the recombinogenic event depends on the exchange of subunits between the two enzyme molecules, followed by ligation of the cleaved nucleic acids (17, 19, 22, 27). However, since the type II enzyme can introduce nicks into the double helix (41–44), and single-stranded breaks are a kinetic intermediate in the double-stranded DNA cleavage/religation reaction of topoisomerase II (4, 42, 45), alternative recombination pathways that do not require two separate enzyme homodimers or subunit exchange also should be considered.

Recent evidence indicates that topoisomerase II can join single-stranded DNA (that results from enzyme-mediated cleavage of aDNA fragment) to an oligonuclease acceptor (40). The present study characterized this intermolecular ligation activity in order to investigate the enzyme’s potential to recombine DNA molecules following the introduction of single-stranded breaks in the nucleic acid backbone. Results indicate that topoisomerase II can mediate illegitimate recombination events, at least in vitro, by a mechanism that does not rely on double-stranded DNA cleavage or subunit exchange.

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells or 6- to 12-h-old embryos, as
described by Shelton et al. (46). All preparations employed were ≥95% homogeneous. Protein concentrations were determined by Bradford analysis using bovine serum albumin as a standard (46). Circular bacteriophage ϕX174 (+) strand DNA and terminal deoxynucleotidyltransferase were from Gibco-BRL; [α-32P]dATP, [γ-32P]ATP, [α-32P]dCTP, [α-32P]dGTP, [α-32P]dTTP, and [γ-32P]ATP (7300 Ci/mmol) were from Amersham Corp.; sodium dodecyl sul fate and proteinase K were from E. Merck Biochemicals; polyethylene glycol 20,000 was from BDH Chemicals; Tris was from Sigma; ATP, dATP, Sephadex G-50 (medium), and bacteriophage T4 polynucleotide kinase were from Pharmacia LKB Biotechnology, Inc.; Taq DNA polymerase, dATP, dCTP, dGTP, and dTTP were from Perkin-Elmer Cetus Instruments; restriction endonucleases EcoRI, PstI, and PvuII were from New England Biolabs and oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. All other chemicals were analytical reagent grade.

Preparation of Double-stranded Oligonucleotide Acceptor Molecules for Intermolecular DNA Ligation Reactions

Blunt-ended 56-mer for Standard Assays—A single-stranded 56-base oligonucleotide with the sequence shown in Fig. 1 (top strand) and its complementary oligonucleotide were radioactively labeled on their 5'-termini in separate reaction mixtures. Reaction mixtures contained 27.5 pmol (0.5 µg) of oligonucleotide (all DNA concentrations are reported as molecules rather than total nucleotides), 15 units of polynucleotide kinase, and 37 pmol of [γ-32P]ATP (3000 Ci/mmol) in a total of 20 µl of kinase buffer supplied by Pharmacia LKB Biotechnology Inc. Following a 2-h incubation at 37°C, reaction mixtures were diluted to 200 µl with the addition of 1 × TE (10 mM Tris-HCl, pH 7.3, and 1 mM EDTA) and filtered through a column of Sephadex G-50 (medium). Phosphorylated oligonucleotides were precipitated with ethanol and dried under partial vacuum as described by Sambrook et al. (47), followed by resuspension in 20 µl of 0.5 × TE. After this procedure, oligonucleotides contained ~2 × 10^6 cpm/µmol. The complementary phosphorylated oligonucleotides were annealed by mixing equimolar amounts, heating to 70°C for 10 min, and cooling to 25°C over a period of 2 h. Samples were diluted with nonlabeled annealed oligonucleotides to a final specific activity of ~2 × 10^6 cpm/µmol blunt-ended double-stranded oligonucleotide prior to intermolecular ligation reactions.

Double-stranded Oligonucleotide Lacking a 3'-OH—Radioactively labeled double-stranded 56-mer (prepared as described above) (2.8 pmol) was incubated with terminal deoxynucleotidyltransferase (30 units) and 1 mM dATP in a total of 17 µl of 100 mM potassium cacodylate, pH 7.2, 2 mM CoCl2, and 0.2 mM diithiothreitol for 2 h at 37°C. The oligonucleotide was phenol-extracted, ethanol-precipitated, resuspended in 10 µl of 0.5 × TE, and diluted with nonradioactive double-stranded 56-mer that lacked a 3'-OH to a final specific activity of ~2 × 10^5 cpm/µmol.

Double-stranded Oligonucleotide Lacking a 5'-Phosphate—The single-stranded 56-base oligonucleotide shown in Fig. 1 (bottom strand) (55 pmol) and an equimolar amount of an oligonucleotide complementary to the EcoRI sites at the 5'-termini of the 20-mer primer were extended by incubating the mixture in an Ericomp Twin Block System thermocycler for 10 cycles (2 min at 95°C, 1.5 min at 50°C, and 2 min at 72°C), followed by a final cycle of 10 min at 72°C. (Cycles beyond the first round were employed to ensure complete extension of the 20-mer.) Reaction products were phenol/ chloroform-extracted, filtered through Sephadex G-50 (medium), and ethanol-precipitated, and resuspended in 200 µl of 0.5 × TE. The resulting radioactive double-stranded 56-mer was either phosphorylated by polynucleotide kinase with nonradioactive ATP as described above (as a control) or subjected to mock phosphorylation in the absence of polynucleotide kinase. Oligonucleotides were recovered by gel filtration and ethanol precipitation as described above, resuspended in 10 µl of 0.5 × TE and further diluted in 0.5 × TE to a final specific activity of ~2 × 10^5 cpm/µmol.

Oligonucleotides with Different End Configurations—Nonradioactive double-stranded 56-mer (220 pmol) was digested with EcoRI (300 units), PstI (300 units), and PvuII (150 units) in a final volume of 200 µl of the appropriate restriction enzyme buffer supplied by New England Biolabs. (See Fig. 1 for the location of restriction endonuclease cleavage sites.) Mixtures were incubated for 2 h at 37°C, followed by phenol extraction, ethanol precipitation, and resuspension in 15 µl of 0.5 × TE. Twenty microilters of loading buffer (80% formamide, 50 mM Tris borate, pH 8.3, 0.005% bromphenol blue, 0.05% xylene cyanol FF) was added, and samples were subjected to electrophoresis in a 7 M urea, 8% polyacrylamide gel in 100 mM Tris borate, pH 8.3, and 2 mM EDTA. The 45-base oligonucleotides that were generated by the restriction digests were excised from the gel, overhanging in overnight in 500 mM Na2HAc, 10 mM MgOAc, and 1 mM EDTA, and purified by column chromatography on Sephadex G-50 (medium). Protein concentrations were determined by Bradford analysis using bovine serum albumin as a standard (46).

Intermolecular DNA ligation by Topoisomerase II

Assays were carried out by a modification of the procedure of Galle and Osheroff (40). Unless otherwise noted, 150 nm topoisomerase II and 5 nm circular ϕX174 (+) strand DNA were incubated at 30°C for 5 min in 18 µl of 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 2.5% glycerol (w/v), and 7.5 mM MgCl2. The [γ-32P]phosphate end-labeled double-stranded 56-mer (25 pmol) and polyethylene glycol 20,000 (5%, w/v) was added to the solution to a final volume of 24 µl. DNA ligation reaction mixtures were further incubated for 60 min at 30°C. Assays were terminated by the addition of 1 µl of 250 mM EDTA, followed by 2 µl of 10% sodium dodecyl sulfate. Proteinase K (2 µl of a 1 mg/ml solution) was added, and topoisomerase II was digested at 37°C for 45 min. Loading buffer (2 µl of 60% sucrose, 0.05% bromphenol blue, 0.35% xylene cyanol FF, and 10 mM Tris-HCl, pH 7.9) was added, and products were subjected to electrophoresis at 10 V/cm in 1.2% agarose (MCB) gels in 100 mM Tris borate, pH 8.3, and 2 mM EDTA. Following electrophoresis, gels were stained in an aqueous solution of ethidium bromide (0.8 µg/ml) and photographed through Kodak 244 and 12 filters with Polaroid type 665 positive/negative film. Under the conditions employed, the intensity of the negative was proportional to the amount of DNA present. Levels of DNA cleavage were monitored by quantitating the percent of circular form ϕX174 (+) strand. The agarose gel was dried and the incorporated radioactivity was visualized by autoradiography with Kodak XAR film and a Du Pont Lightening Plus screen. Levels of intermolecular DNA ligation were monitored by quantitating the [γ-32P]phosphate incorporated into the uncleaved linear ϕX174 DNA band. DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak 244 and 12 filters with Polaroid type 665 positive/negative film. Under the conditions employed, the intensity of the negative was proportional to the amount of DNA present. Levels of DNA ligation were normalized relative to reactions that were incubated for 60 min and contained 5 nm ϕX174 DNA and 25 nm oligonucleotide.

Southern Analysis of Intermolecular DNA Ligation Products

Following intermolecular DNA ligation using nonlabeled double-stranded 56-mers, samples were subjected to electrophoresis, and the DNA bands were visualized as described above. The DNA was transferred from the agarose gel to a GeneScreen Plus hybridization transfer membrane (New England Nuclear Research products) and analyzed by Southern blot hybridization according to the procedures described in Sambrook et al. (47). A radioactively labeled 20-base oligonucleotide with the sequence AGACG/TTCCT/GACGCT/
GGCGCA (complementary to residues 155–174 of φX174 (+) strand DNA) was used to probe for φX174 molecules, and each strand of the 56-mer was used to probe for its complementary strand.

Separation of Intermolecular DNA Ligation Reaction Products by Alkaline Gel Electrophoresis

Blunt-ended double-stranded 56-mers in which one or the other strand was [32P]phosphate end-labeled were prepared as described above and employed as acceptor molecules in intermolecular ligation reactions. DNA products were ethanol-precipitated, resuspended in 20 μl of 100 mM NaOH and 1 mM EDTA, and subjected to electrophoresis in an alkaline gel (1.2% agarose gel formed in 100 mM Tris borate, pH 8.3, and 2 mM EDTA and soaked overnight in alkaline running buffer, 30 mM NaOH, and 1 mM EDTA). The agarose gel was dried, and the radioactivity was visualized by autoradiography as described above.

Determination of Steady-state Levels of Unit Length Linear φX174 DNA Cleavage Product Generated during Intermolecular Ligation Reactions

Prior to assays, the linear contaminant (~15% of the total DNA) present in preparations of φX174 (+) strand DNA substrate (6 pmol of total DNA) was radioactively labeled as described above on its 5' terminus with 15 units of polynucleotide kinase and 17 pmol of [γ-32P]ATP (~3000 Ci/mmol) in a total of 56 μl of kinase buffer. Following ethanol precipitation, samples were resuspended in 20 μl of 0.5 x TE and diluted with nonlabeled φX174 (+) strand DNA to a final specific activity of ~3.5 x 106 cpm/pmol. Intermolecular DNA ligation assays were carried out as described above using nonlabeled double-stranded 56-mer and analyzed by agarose gel electrophoresis. The percent of DNA cleavage and the total amount of unit length linear φX174 DNA present were determined by scanning densitometry. The amount of unit length linear contaminant in each sample was determined by autoradiography. Levels of unit length linear cleavage product generated by topoisomerase II-mediated DNA cleavage during intermolecular ligation were calculated by subtracting the amount of unit length linear contaminant from the total amount of unit length linear φX174 molecules present at each time point.

RESULTS

Intermolecular DNA Ligation Activity of Topoisomerase II—Central to topoisomerase II-mediated nucleic acid recombination is the enzyme's ability to carry out an intermolecular DNA ligation event. Recently, a novel assay was developed to monitor this enzyme activity (40). Although it was demonstrated that the intermolecular DNA ligation reaction is intrinsic to topoisomerase II, the properties of this potentially important activity were not determined.

In order to investigate potential roles for topoisomerase II in the recombination process, the system of Gale and Osheroff (40) was employed to characterize the enzyme's intermolecular DNA ligation reaction. In this assay, topoisomerase II is incubated with circular bacteriophage φX174 (+) strand molecules for 5 min to generate covalent enzyme-cleaved linear DNA intermediates. Following this initial incubation, a 5-fold molar excess (with respect to φX174 DNA) of a radioactively labeled double-stranded oligonucleotide acceptor molecule is added to the reaction mixture. An autoradiogram showing a representative time course for the ligation of cleaved φX174 DNA to a 56-mer acceptor oligonucleotide by topoisomerase II can be seen in Fig. 2. Intermolecular ligation results in a shift in the electrophoretic mobility of labeled oligonucleotide to the position of linear φX174 molecules. As expected for this enzyme-mediated activity, no radioactivity was observed at the position of circular uncleaved bacteriophage DNA.

Intermolecular DNA ligation proceeded in a time-dependent fashion. At longer reaction times, some of the radioactive label was incorporated into smaller than unit length linear φX174 (+) strand fragments. These fragments result from multiple topoisomerase II cleavage events on a single bacteriophage substrate (40). For the purpose of this study, levels of intermolecular DNA ligation were monitored by quantitating the label incorporated into unit length linear φX174 molecules. To normalize data between independent assays, the amount of label incorporated under standard conditions (60 min incubation, 5 nM φX174 (+) strand DNA, 25 nM double-stranded 56-mer oligonucleotide), as quantitated by scanning densitometry of autoradiograms, was arbitrarily assigned a value of 1.0. As determined by liquid scintillation counting of excised DNA bands (48), 2.0–2.5% of the cleaved unit length linear bacteriophage DNA was ligated to the labeled 56-mer acceptor molecule (see Fig. 1) in standard assays. Levels that exceeded 4.2% were observed under some conditions. These levels of intermolecular ligation are similar to those obtained when a 42-mer oligonucleotide acceptor was employed previously (40).

The efficiency of topoisomerase II-mediated intermolecular ligation observed when φX174 (+) strand DNA was used as the initial cleavage substrate is considerably higher than that obtained using double-stranded nucleic acid as substrates. For example, when double-stranded bacteriophage λ was employed, levels of intermolecular ligation (determined by a genetic screen of reaction products) ranged from 0.01 to 0.15% (17, 19). In the present system, no ligation was observed (either in the absence or presence of ATP) when double-stranded pBR322 plasmid molecules were utilized as cleavage substrates (not shown). The relatively high efficiency observed with φX174 (+) strand molecules most likely reflects the fact that the single-stranded 3'-OH DNA terminus generated upon cleavage can dissociate from the active site of topoisomerase II (40). This dissociation disrupts the enzyme's normal intramolecular DNA cleavage/relinkage equilibrium (established with double-stranded substrates) and allows a separate 3'-OH terminus to invade the topoisomerase II-DNA cleavage complex.

The bimolecular nature of intermolecular ligation allows...
the affinity of topoisomerase II for its acceptor oligonucleotide to be determined. This was accomplished by analyzing the concentration dependence of the 56-mer in ligation reactions. As seen in Fig. 3, levels of intermolecular ligation increased with the oligonucleotide concentration. The apparent $K_m$ of topoisomerase II for the 56-mer was $\sim 20$ nM, as assessed by Eadie-Hofstee (Fig. 3, inset) or Lineweaver-Burk (not shown) transformation (49) of the data. This value is consistent with previously derived affinity constants determined for binding interactions of the enzyme with double-stranded DNA (50).

Covalent Linkage of $\phi X174$ DNA to the Acceptor Oligonucleotide Following Topoisomerase II-mediated Intermolecular Ligation—Two independent techniques were employed to confirm the covalent linkage between $\phi X174$ DNA and the acceptor 56-mer following intermolecular ligation. First, reaction products from assays that employed nonradioactive oligonucleotide were examined by Southern hybridization analysis (Fig. 4, right) using DNA probes specific for either the $\phi X174$ molecule (lanes 1–5) or the top strand (see Fig. 1) of the 56-mer (lanes 6–10). In all cases, the oligonucleotide comigrated with $\phi X174$ DNA at positions where ligation products were observed (i.e. unit-length and sub-unit-length linear bacteriophage DNA). No oligonucleotide comigrated with uncleaved circular $\phi X174$ molecules. Identical results were obtained when blots were examined with a probe specific for the bottom strand of the 56-mer (not shown).

Second, intermolecular DNA ligation products were analyzed by alkaline gel electrophoresis (Fig. 4, left). For these experiments, either the top strand (not shown) or the bottom strand (see Fig. 1) of the 56-mer (lanes 1–3) was radioactively labeled. As found with the Southern hybridization analysis, the oligonucleotide comigrated with linear, but not with uncleaved circular bacteriophage DNA. Identical results were obtained when either strand of the oligonucleotide acceptor molecule was labeled. These data provide strong evidence that topoisomerase II-mediated intermolecular ligation results in the covalent joining of cleaved $\phi X174$ molecules to the 56-mer.

Upon cleavage of either double- (1, 2, 4, 51, 52) or single-stranded DNA (40, 53), topoisomerase II forms a covalent bond with the newly generated 5'-phosphate termini. Thus, the enzyme would be expected to link cleaved $\phi X174$ DNA to the 3'-OH termini (rather than the 5'-phosphate termini) of the acceptor oligonucleotide.1 To determine the requirement for the 3'-OH termini of the 56-mer, a labeled oligonucleotide that lacked its terminal hydroxyl groups was generated. This was accomplished by incubating the 56-mer with terminal deoxynucleotidyltransferase and ddATP. When the deoxyoligonucleotide was employed as the acceptor molecule in assays, levels of intermolecular ligation decreased by more than 85% (Fig. 5). The low level of activity remaining probably reflects the incomplete incorporation of ddAMP into the oligonucleotide. However, to ensure that intermolecular ligation was always between the 5'-phosphate terminus of cleaved $\phi X174$ DNA and the 3'-OH termini of the 56-mer, labeled oligonucleotide acceptor molecules that lacked a 5'-phosphate were generated as described under “Experimental Procedures.” In two independent experiments, levels of intermolec-

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1 This result is opposite to that expected if intermolecular ligation were mediated by a contaminating DNA ligase. In this case, topoisomerase II blocks the 5'-terminus of cleaved $\phi X174$ DNA, only the newly generated free 3'-OH terminus would be available for ligation to the 5'-phosphate termini of the oligonucleotide acceptor.
ular DNA ligation to the 5'-OH-containing oligonucleotide were at least as high as those obtained in the presence of the 5'-phosphate-containing acceptor (not shown). Therefore, as expected, topoisomerase II covalently joins cleaved \( \phi X174 \) DNA to the 3'-OH terminus of the oligonucleotide acceptor.

Due to interactions between topoisomerase II and the agarose gel matrix, the electrophoretic mobility of DNA to which it is covalently attached is severely retarded (42, 54). Normal DNA electrophoretic patterns are reestablished following digestion of the bound enzyme with proteinase K (42, 54). In contrast to cleaved (but unligated) \( \phi X174 \) molecules (40), the mobility of ligation products did not change when the proteinase K digestion step was omitted (not shown). This indicates that the covalent phosphotyrosine bond established between topoisomerase II and the 5'-terminus of cleaved \( \phi X174 \) DNA is displaced by the 3'-OH of the acceptor oligonucleotide.

Effects of Salt, Divalent Cation, and ATP on Intermolecular DNA Ligation—Since salt, divalent cation, and ATP all affect the DNA cleavage/religation equilibrium of topoisomerase II (50-52, 54, 55), their effects on the enzyme's intermolecular DNA ligation reaction were characterized. However, if conclusions concerning the influence of these compounds on intermolecular ligation are to be valid, it is first necessary to control for their effects on DNA cleavage. This was accomplished by analyzing levels of unit length linear \( \phi X174 \) DNA cleavage intermediates produced over the course of a ligation assay.

Unit length linear \( \phi X174 \) DNA is generated by topoisomerase II-mediated cleavage of circular substrates and is depleted when additional enzyme molecules convert unit length DNA to sub-unit length fragments (40). Since both the generation and the depletion of unit length cleavage intermediates are catalyzed by the same enzyme-mediated reaction, once a steady state is attained, levels of unit length cleavage intermediates available for ligation should remain constant. Therefore, the effects of compounds (even those that block the enzyme's cleavage reaction) on intermolecular DNA ligation can be assessed as long as they are added to assay mixtures during this steady-state window.

As seen in Fig. 6, a steady-state level of unit length linear cleavage intermediate was established during the first 5 min of intermolecular ligation. (Levels of cleavage intermediate present at time 0 were generated during the 5-min preincubation of topoisomerase II with \( \phi X174 \) (+) strand molecules in the absence of the 56-mer.) The concentration of cleaved unit length \( \phi X174 \) DNA is maintained through the 60-min ligation time point, after which the amount of circular bacte-

![Fig. 6. Steady-state analysis of unit length linear cleaved \( \phi X174 \) (+) strand DNA formed during intermolecular ligation. A time course for intermolecular DNA ligation is shown. Assays were carried out, and percentages of circular \( \phi X174 \) (+) strand substrate (open circles) and unit length linear \( \phi X174 \) DNA cleavage products (closed circles) were determined as described under "Experimental Procedures."](image)

![Fig. 7. Effect of salt on intermolecular DNA ligation mediated by topoisomerase II.](image)

Although topoisomerase II does not require a divalent cation for noncovalent interactions with DNA (50, 56, 57), a divalent cation is necessary for enzyme-mediated cleavage of \( \phi X174 \) (+) strand molecules (40), as well as for cleavage and intermolecular religation of double-stranded nucleic acids (51, 54, 55). The enzyme's requirement for a divalent cation for intermolecular ligation was determined by chelating the magnesium in assays with EDTA. As seen in Fig. 7, EDTA completely inhibited the joining of \( \phi X174 \) DNA to the 56-mer. This inhibition was due to the chelation of the divalent cation rather than to the presence of EDTA, per se, since the back addition of excess MgCl\(_2\) once again supported ligation (not shown). Therefore, the intermolecular DNA ligation reaction of topoisomerase II requires the presence of a divalent cation.

Neither the intramolecular religation of double-stranded DNA (51, 54, 55) nor the intermolecular joining of cleaved \( \phi X174 \) (+) strand molecules to the 56-mer depends on the presence of ATP. Since in vitro recombination of double-stranded DNA substrate becomes too low to support this steady state. Thus, all of the effectors discussed below were added to assays after 10 min of intermolecular DNA ligation had taken place.

Although noncovalent topoisomerase II-DNA interactions are disrupted by increased salt concentrations (>95% dissociation at 0.5 mM NaCl) (50), the enzyme's intramolecular double-stranded DNA religation activity is not sensitive to salt at concentrations up to 1 M (42, 52, 54). As expected for a reaction that requires both topoisomerase II-DNA binding and ligation, the intermolecular joining of cleaved \( \phi X174 \) (+) strand DNA to the oligonucleotide acceptor was blocked following the addition of 0.5 mM NaCl to assays (Fig. 7).

![Fig. 8. Requirement of intermolecular DNA ligation for a divalent cation. Time courses were carried out in the presence of 7.5 mM MgCl\(_2\) as described under "Experimental Procedures." Following 10 min of intermolecular DNA ligation (denoted by the arrow), either H\(_2\)O (open circles) or NaCl (500 mM final concentration, closed circles) was added to assay mixtures.](image)
Stranded bacteriophage λ DNA by calf thymus topoisomerase II is stimulated ~10-fold by ATP (19), the effects of the high energy cofactor on intermolecular ligation of single-stranded φX174 DNA to the oligonucleotide acceptor by the Drosophila enzyme was determined. As shown in Fig. 9, no stimulation of intermolecular DNA ligation was observed in the presence of ATP.

Characterization of the Oligonucleotide Acceptor—As determined by analysis of ligation products on denaturing alkaline agarose gels (see Fig. 4), topoisomerase II joined cleaved φX174 (+) strand DNA to either strand of the blunt-ended double-stranded 56-mer with equal efficiency. To examine the influence of the configuration around the 3′-OH termini of oligonucleotide acceptor molecules, the 56-mer was digested at the restriction endonuclease sites shown in Fig. 1. Digestion with PvuII, EcoRI, or PstI yielded double-stranded 45-mers that contained a blunt end, a 4-base 5′-overhang, or a 4-base 3′-overhang, respectively. Topoisomerase II utilized all three 45-mers as acceptor molecules for intermolecular DNA ligation with similar efficiencies (Table I). Although the enzyme displayed a slight preference for the 45-mer with a 5′-overhang over that with a blunt end over that with a 3′-overhang, it is not clear whether the minor differences in activity are due solely to changes in the oligonucleotide end configuration or are also influenced by differences in the DNA sequence around the ends.

Three additional aspects of the oligonucleotide acceptor were examined (not shown). First, intermolecular DNA ligation was not enhanced by the inclusion of a strong topoisomerase II recognition/cleavage site (51) in the double-stranded acceptor molecule. Second, the enzyme was able to utilize single-stranded oligonucleotides as acceptors for intermolecular ligation. Finally, topoisomerase II was able to transfer cleaved φX174 DNA to a 3′-OH at a nick or a 5-base gap, but with a reduced efficiency.

**DISCUSSION**

A number of studies implicate a role for topoisomerase II in mediating DNA recombination in vitro and in vivo (17, 19, 21, 26). All of the models that have been proposed for this activity require enzyme-mediated double-stranded DNA cleavage, followed by subunit exchange between two separate homodimers of topoisomerase II (17, 19, 22, 29). However, since the type II enzyme can introduce nicks (that are kinetic intermediates in its double-stranded DNA cleavage/religation pathway) into the double helix (41-44), alternative mechanisms for illegitimate recombination that rely on the single-stranded DNA cleavage activity of topoisomerase II should also be considered. The intermolecular DNA ligation mechanism that was addressed in the present study is similar to models proposed for topoisomerase I-mediated recombination (16, 22, 24), does not require subunit exchange, and relies on the actions of a single topoisomerase II homodimer.

In order to examine the potential for this alternative illegitimate recombination pathway, the properties of topoisomerase II-mediated intermolecular DNA ligation were characterized. To this end, a model system in which the enzyme covalently joins cleaved φX174 (+) strand DNA to a double-stranded oligonucleotide acceptor was employed. Results indicate that topoisomerase II ligates the cleaved bacteriophage DNA exclusively to the 3′-OH termini of acceptor molecules in a time-dependent and an oligonucleotide concentration-dependent fashion. As previously determined for the enzyme’s intramolecular DNA religation activity (51, 54, 55), intermolecular ligation requires a divalent cation. In contrast to recombination of double-stranded DNA substrates reported for calf thymus topoisomerase II (19), the intermolecular ligation of cleaved single-stranded φX174 DNA to an oligonucleotide acceptor is not stimulated by the presence of ATP. This finding demonstrates that the enzyme’s DNA strand

**FIG. 10. DNA structures that could generate potential substrates for illegitimate recombination mediated by topoisomerase II.** The homodimeric enzyme is represented by the paired ovals. A, topoisomerase II-mediated cleavage in single-stranded regions of DNA. B, cleavage across a pre-existing nick in the DNA backbone. C, cleavage at a double-stranded/single-stranded DNA junction present at the base of a hairpin loop (or a Holliday junction). D, cleavage in double-stranded DNA that is subsequently converted to a single-stranded DNA region by the actions of a helicase or other replication machinery.
Transcription complexes (3), or replication complexes (63). During DNA replication on the lagging strand of parental DNA (63), covalent cleavage of DNA by topoisomerase II generates such a diffusible 3'-OH terminus upon cleavage by the Drosophila enzyme (40) and, as predicted, is an excellent model substrate for illegitimate recombination.

Three different nucleic acid structures have been shown to produce diffusible 3'-OH termini following topoisomerase II-mediated cleavage: single-stranded DNA, duplex molecules that contain nicks adjacent to the enzyme's cleavage site, and duplex molecules with cleavage sites at double-stranded/single-stranded junctions (53, 58). Recent evidence indicates that nucleic acid molecules with this latter structure in fact can serve as substrates for a topoisomerase II-mediated molecular DNA ligation reaction (58). Although sites of 4x174 (+) strand DNA cleavage by the bacteriophage 4x174 topoisomerase II cleaves 4x174 DNA in single-stranded regions and in regions that contain double-stranded/single-stranded junctions (i.e. at the base of hairpin loops) (53).

The nucleic acid structures described above, as well as others that also have potential to generate diffusible 3'-OH termini, all exist in vivo (Fig. 10). For example, single-stranded regions (Fig. 10A) are produced in the DNA helix by the actions of damaging agents (61), repair enzymes (61, 62), and replication on the lagging strand of parental DNA (63). Double-stranded/single-stranded junctions (Fig. 10, C and D) are formed by the conversion of palindromic sequences to cruciforms (64), the production of homologous recombination intermediates (65), and gapped DNA formed during lagging strand DNA replication (63). Finally, duplex nucleic acids (undergoing cleavage by topoisomerase II) may be converted to single-stranded molecules by the actions of helicases (66), transcription complexes (3), or replication complexes (63).

The present study describes a novel mechanism for illegitimate recombination that relies on the ability of a single topoisomerase II homodimer to mediate intermolecular DNA ligation. The fact that DNA structures necessary to generate appropriate recombination substrates are prevalent in vivo suggests that this intermolecular DNA ligation model for topoisomerase II-mediated recombination has physiological significance.

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