Uncoupling the DNA Cleavage and Religation Activities of Topoisomerase II with a Single-Stranded Nucleic Acid Substrate: Evidence for an Active Enzyme-Cleaved DNA Intermediate†

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ABSTRACT: Following its cleavage of double-stranded DNA, topoisomerase II is covalently bound to the 5′-termini of both nucleic acid strands. However, in order to isolate this enzyme–cleaved DNA complex in the presence of magnesium (the enzyme's physiological divalent cation), reactions must be terminated by the addition of a strong protein denaturant such as sodium dodecyl sulfate (SDS). Because of the requirement for a protein denaturant, it is unclear whether DNA cleavage in this in vitro system takes place prior to or is induced by the addition of SDS. To distinguish between these two possibilities, experiments were carried out to determine whether topoisomerase II bound DNA contains 3′-OH termini prior to denaturation. This was accomplished by using circular single-stranded *ϕX174* DNA as a model substrate for the enzyme. As found previously for topoisomerase II mediated cleavage of double-stranded DNA, the enzyme was covalently linked to the 5′-termini of cleaved *ϕX174* molecules. Moreover, optimal reaction pH as well as optimal salt and magnesium concentrations was similar for the two substrates. In contrast to results with double-stranded molecules, single-stranded DNA cleavage increased with time, was not salt reversible, and did not require the presence of SDS. Furthermore, cleavage products generated in the absence of protein denaturant could be labeled at their 3′-OH DNA termini by incubation with terminal deoxynucleotidyltransferase and [α-^{32}P]ddATP. Finally, cleaved *ϕX174* molecules could be joined to a radioactively labeled double-stranded oligonucleotide by a topoisomerase II mediated intermolecular ligation reaction. These results demonstrate that single-stranded DNA is cleaved by the enzyme prior to the addition of SDS and strongly suggest that the covalent topoisomerase II–cleaved DNA complex observed in vitro is an active intermediate in the enzyme's catalytic cycle.

Topoisomerase II is a ubiquitous enzyme that catalyzes alterations in the topological structure of DNA (Wang, 1985; Vosberg, 1985; Osheroff, 1989a). The enzyme is essential for the viability of eukaryotic cells (Goto & Wang, 1984; DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985) and plays a critical role in chromosome segregation (Uemura & Yanagida, 1984, 1986; DiNardo et al., 1984; Holm et al., 1985, 1989, Uemura et al., 1987; Rose et al., 1990). In addition, topoisomerase II is important for the maintenance of chromosome structure (Earnshaw et al., 1985; Earnshaw & Heck, 1985; Berrios et al., 1985; Gasser et al., 1986; Gasser & Laemmli, 1986; Cockerill & Garrard, 1986; Uemura et al., 1987; Adachi et al., 1989; Sperry et al., 1989) and may also be involved in DNA replication (Wang, 1985; Vosberg, 1985; Snapka, 1986; Yang et al., 1987; Brill et al., 1987; Osheroff, 1989a, Kim & Wang, 1989b; Annunziato, 1989; Schaack et al., 1990) and recombination (Bae et al., 1988; Christman et al., 1988; Kim & Wang, 1989a; Sperry et al., 1989; Dillehay et al., 1989; Rose et al., 1990).

Topoisomerase II functions by cleaving both strands of a DNA double helix, passing an intact double helix through this break, and religating the broken strands (Wang, 1985; Vosberg, 1985; Osheroff, 1989a). This ability to introduce transient breaks in the DNA backbone is critical to the enzyme's physiological functions. In addition, the DNA cleavage/religation cycle of topoisomerase II appears to be the target for a number of clinically relevant antineoplastic agents (Glisson & Ross, 1987; D'Arpa & Liu, 1989; Zwelling, 1989). The chemotherapeutic efficacies of these drugs correlate (at least in part) with their abilities to inhibit the enzyme's religation of cleaved DNA (Glisson & Ross, 1987; D'Arpa & Liu, 1989; Zwelling, 1989; Osheroff, 1989b; Robinson & Osheroff, 1990).

A hallmark of the topoisomerase II mediated cleavage reaction is the covalent linkage between the enzyme and both of the newly generated 5′-termini of cleaved double-stranded DNA (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989). Since the ends of the DNA are not released from the enzyme's active site prior to religation, it has been difficult to detail the mechanism of this reaction. In order to monitor DNA cleavage in vitro, topoisomerase II is incubated with double-stranded molecules in the presence of a divalent cation (Sander & Hsieh, 1983; Osheroff, 1987). Cleavage products are observed following the rapid addition of a protein denaturant such as sodium dodecyl sulfate (SDS)[1] (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff, 1987). The cleavage of double-stranded DNA

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MES, 2-(*N*-morpholino)-ethanesulfonic acid; AMPSO, 3-[*N*-(α,α-dimethylhydroxyethyl)-amino]-2-hydroxypropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; PEG, poly(ethylene glycol).

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appears to be an equilibrium process; reactions are time independent (beyond 5 s) and can be reversed by the addition of salt (Liu et al., 1983; Osheroff & Zechiedrich, 1987; Osheroff, 1989a; Zechiedrich et al., 1989). However, because of the requirement for SDS in the in vitro system, it is not clear whether the denaturant interrupts the equilibrium by trapping a steady-state enzyme complex in which the DNA is already cleaved or by inducing the enzyme in a precleavage complex to hydrolyze the DNA backbone (Liu et al., 1983; Wang, 1985; Vosberg, 1985; Osheroff, 1989a).

To distinguish between these two possibilities, the cleavage of single-stranded DNA by *Drosophila melanogaster* topoisomerase II was used as a model system. Since the enzyme should covalently attach only to the single 5′-terminus generated during cleavage, the newly formed 3′-OH terminus may be able to diffuse away from the active site of topoisomerase II. Such an event would uncouple the enzyme's cleavage/religation cycle that occurs with double-stranded DNA. Thus, if cleavage in the in vitro assay takes place prior to the addition of SDS, the topoisomerase II mediated reaction with single-stranded DNA should be time dependent, should not be salt reversible, and should provide a 3′-OH terminus that can be labeled with terminal deoxynucleotidyltransferase. In contrast, if cleavage is induced only following the addition of SDS, results should reflect the characteristics of an equilibrium process as described above.

The present study indicates that topoisomerase II mediated cleavage of single-stranded DNA takes place prior to the addition of SDS in the in vitro system. In addition, the topoisomerase II–cleaved DNA complex is kinetically competent as demonstrated by the enzyme's ability to ligate cleaved single-stranded molecules to a double-stranded oligonucleotide.

A preliminary report of some of this work has appeared (Gale & Osheroff, 1989).

Experimental Procedures

D. melanogaster DNA topoisomerase II was purified from the nuclei of Kc tissue culture cells or 6–12-h-old embryos as described by Shelton et al. (1983). Circular bacteriophage *φX174* (+) strand DNA, terminal deoxynucleotidyltransferase, and 5× tailing buffer were from BRL; [α-^{32}P]ddATP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham; PEG 20 000 was from BDH Chemicals; Tris and insolubilized calf alkaline phosphatase were from Sigma; bacteriophage T4 polynucleotide kinase was from Pharmacia; MES and AMPSO were from Research Organics; SDS and proteinase K were from E. Merck Biochemicals; and a blunt-ended 42-bp oligonucleotide with the sequence AC-AAT/AGCGT/AATGC/GAATA/TTGAC/ATGCG/TAGCA/TACGA/AA was the generous gift of R. Hamilton and Dr. R. S. Lloyd. All other chemicals were analytical reagent grade.

Cleavage of Single-Stranded DNA by Topoisomerase II. Unless otherwise noted, the standard cleavage assay contained 75 nM topoisomerase II and 5 nM circular *φX174* (+) strand DNA in a total of 20 μL of cleavage buffer (10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 2.5% glycerol (w/v), and 7.5 mM $MgCl_2$). Cleavage was carried out at 30 °C for 60 min and was stopped by the addition of 1 μL of 10% SDS followed by 2 μL of 250 mM EDTA. Proteinase K (2 μL of a 1 mg/mL solution) was added and topoisomerase II was digested at 45 °C for 45 min. Samples were heated at 70 °C for 2 min prior to the addition of 2 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris-HCl, pH 7.9). 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). DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak 24A and 12 filters with Polaroid Type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Biomed Instruments Model SL-504-XL scanning densitometer. Under the conditions employed, the intensity of the negative was directly proportional to the amount of DNA present. The percent of cleavage was estimated by the percent of circular form DNA lost.

Cleavage of Double-Stranded DNA by Topoisomerase II. Assays contained 75 nM topoisomerase II and 5 nM double-stranded pBR322 plasmid (Bolivar et al., 1977) DNA and were carried out as described by Osheroff (1987). Cleavage was determined by the increase in linear molecules.

Labeling the Newly Generated 3′-OH DNA Termini Following Topoisomerase II Mediated Cleavage of Single-Stranded φX174 Molecules. Topoisomerase II (75 nM) and 5 nM circular *φX174* (+) strand DNA were incubated at 30 °C for 0–90 min in 18 μL of cleavage buffer. Newly generated 3′-OH DNA termini were labeled at 30 °C for 30 min following the addition of terminal deoxynucleotidyltransferase and tailing buffer (100 mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM dithiothreitol final concentration) which contained dTTP (1 mM final concentration) and [α-^{32}P]-ddATP (~3 × 10^6 cpm/assay) to a final volume of 25 μL. Samples were processed by one of the following two methods:

(1) Reactions were terminated by the addition of 1 μL of 10% SDS. EDTA (2 μL of a 250 mM solution) was added and samples were digested with proteinase K as described above. DNA products were ethanol precipitated to remove unincorporated [α-^{32}P]ddATP as described by Sambrook et al. (1989). Samples were resuspended in 25 μL of 200 mM NaCl and analyzed by electrophoresis as described above. The agarose gel was dried and the incorporated radioactivity was visualized by autoradiography with Kodak XAR film and a Du Pont Lightning Plus screen.

(2) Reaction products were spotted onto Whatman GF/C filters, and the filters were immediately dropped into 100 mL of 0.1 M sodium pyrophosphate in 20% TCA on ice. Filters were washed three times in 100 mL of 0.1 M sodium pyrophosphate in 20% TCA, once in 200 mL of 70% (v/v) ethanol, and once in 200 mL of 95% (v/v) ethanol. All washes were for 10 min with swirling on ice. Filters were dried, and the amount of [α-^{32}P]ddAMP incorporated was quantitated with a Beckman LS-7500 liquid scintillation counter and Ecolume (ICN) aqueous counting scintillant.

Preparation of Oligonucleotides for Intermolecular Ligation Reactions. A single-stranded 42-base oligonucleotide with the sequence ACAAT/AGCGT/AATGC/GAATA/TTGAC/ATGCG/TAGCA/TACGA/AA and its complementary oligonucleotide were radioactively labeled on their 5′-termini in separate reaction mixtures. Reaction mixtures contained 0.5 μg (37 pmol) of oligonucleotide, 15 units of polynucleotide kinase, and 40 pmol of [γ-^{32}P]ATP (~3000 Ci/mmol) in a total of 20 μL of kinase buffer supplied by Pharmacia. Following a 2-h incubation at 37 °C, reaction mixtures were diluted to 200 μL by the addition of 10 mM Tris-HCl, pH 7.9, and 1 mM EDTA and filtered through Sephadex G-50. Phosphorylated oligonucleotides were precipitated with ethanol, dried under partial vacuum, and redissolved in 20 μL of 5 mM Tris, pH 7.4, and 0.5 mM EDTA. After this procedure, oligonucleotides contained ~2 × 10^6 cpm/pmol. The com-

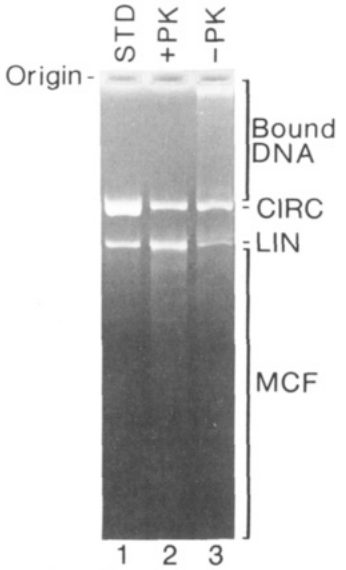

FIGURE 1: Cleavage of *ϕX174* (+) strand DNA by *D. melanogaster* topoisomerase II. Assays were carried out as described under Experimental Procedures. An agarose gel is shown. Lane 1, *ϕX174* (+) strand DNA standard; lane 2, DNA cleavage products digested with proteinase K (+PK); lane 3, DNA cleavage products not digested with proteinase K (−PK). The positions of circular (CIRC), unit-length linear (LIN), and topoisomerase II bound *ϕX174* (+) strand DNA are shown. MCF represents multiply cleaved fragments (i.e., less than unit-length linear fragments) of *ϕX174*.

plementary phosphorylated oligonucleotides were annealed by mixing equimolar amounts of each, heating to 95 °C for 10 min, and allowing the solution to cool to 25 °C over a period of 2 h. When necessary, samples were diluted with nonlabeled annealed oligonucleotides to a final specific activity of $\sim 2 \times 10^5$ cpm/pmol double-stranded oligonucleotide prior to intermolecular ligation reactions.

Intermolecular DNA Ligation by Topoisomerase II. Topoisomerase II (150 nM) and 5 nM circular *ϕX174* (+) strand DNA were incubated at 30 °C for 5 min in 18 μL of cleavage buffer. The [^{32}P]phosphate-end-labeled double-stranded 42-bp oligonucleotide (prepared as described above, 25 nM final concentration) and PEG 20000 [5% (w/v) final concentration] were then added to the solution to a final volume of 30 μL. DNA ligation reaction mixtures were incubated for 0–120 min at 30 °C. Assays were terminated and processed as described in procedure 1 of the section on labeling 3′-OH termini.

Results

Previous studies employed single-stranded nucleic acids as model substrates to investigate the DNA cleavage mechanism of topoisomerase I (Been & Champoux, 1980, 1981; Halligan et al., 1982; Tse-Dinh, 1986). In all cases, it was demonstrated that cleavage preceded the addition of SDS in vitro and that the topoisomerase I–cleaved DNA complex was an active reaction intermediate. The use of single-stranded molecules for these experiments was justified by the fact that the type I enzyme acts by making a transient single-stranded nick in the DNA double helix (Wang, 1985; Vosberg, 1985; Osheroff, 1989a).

Kreuzer (1984) found that the type II enzyme from bacteriophage T4 was able to mediate cleavage of single-stranded DNA. Moreover, recent evidence indicates that topoisomerase II cleaves/religates double-stranded DNA by making two sequential single-stranded nicks in the nucleic acid backbone (Muller et al., 1988; Zechiedrich et al., 1989; Anderson et al., 1989; Lee et al., 1989). Therefore, it is likely that the interaction between topoisomerase II and single-stranded DNA may also serve as a useful model to investigate the cleavage mechanism of the type II enzyme.

Table I: Covalent Attachment of Topoisomerase II to the 5′-Terminus of Cleaved ϕX174 (+) Strand DNA[a]

reaction condition	5′-termini/reaction[b] (fmol)	5′-termini labeled/reaction (fmol)
linear pBR322 DNA standard	100	86
ϕX174 (+) strand DNA, no topoisomerase II	22[c]	19
ϕX174 (+) strand DNA + topoisomerase II[d]	>70[e]	18

[a] Following the initial cleavage incubation at 30 °C for 60 min, samples were heated to 75 °C for 10 min, treated with 0.2 unit of insolubilized alkaline phosphatase at 25 °C for 30 min (Ackerman & Osheroff, 1989), and labeled with 5 units of polynucleotide kinase and [γ-^{32}P]ATP (8 pmol, $\sim 1.6 \times 10^7$ cpm) at 37 °C for 30 min. Reactions were terminated by heating to 70 °C for 10 min. Radioactive incorporation was analyzed by TCA precipitation onto GF/C filters and scintillation counting as described under Experimental Procedures. [b] Samples contained 100 fmol of DNA strands per 20-μL assay. [c] This value represents the amount of contaminating linear ϕX174 (+) strand present in the circular DNA preparation employed for this experiment. [d] Sample contained 1.5 pmol of *Drosophila* topoisomerase II in the initial 20-μL incubation mixture. [e] The concentration of 5′-termini was estimated by the loss of circular ϕX174 DNA substrate. Since some ϕX174 (+) strand circles were multiply cleaved by the enzyme during the initial incubation, this value represents the minimum level of 5′-termini generated.

Topoisomerase II Mediated Cleavage of Single-Stranded DNA. To monitor the cleavage of single-stranded nucleic acids by topoisomerase II, the enzyme from *D. melanogaster* was incubated with circular bacteriophage *ϕX174* (+) strand DNA. As seen in Figure 1, topoisomerase II converted the circular *ϕX174* DNA to unit-length and smaller linear fragments. Unit-length linear products represent *ϕX174* strands that were cleaved by a single enzyme molecule, while the smaller DNA fragments were produced by multiple cleavage events. The presence of these multiply cleaved *ϕX174* strands makes it difficult to determine the actual number of cleavage events that occur per assay. Hence, levels of topoisomerase II mediated cleavage in this study were estimated by quantitating the loss of circular *ϕX174* DNA.

A hallmark of topoisomerase II mediated double-stranded DNA cleavage is the covalent attachment of the enzyme to the 5′-termini of its linear and nicked DNA products (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987; Muller et al., 1988; Zechiedrich et al., 1989; Lee et al., 1989). The enzyme was also covalently bound to cleaved single-stranded *ϕX174* molecules (Figure 1). Even after treatment with 0.5% SDS (final concentration) at 70 °C, proteinase K digestion was required to release cleaved *ϕX174* products (compare lanes 2 and 3). Treatment with either SDS or heat readily disrupts noncovalent interactions between topoisomerase II and DNA (Osheroff & Zechiedrich, 1987).

If topoisomerase II is covalently attached to the 5′-termini of cleaved *ϕX174* (+) strands, the 5′-termini should not be available for phosphorylation even after phosphatase treatment. To determine if this was the case, cleavage reactions were stopped by heating, and mixtures were treated with insolubilized alkaline phosphatase and incubated with polynucleotide kinase and [γ-^{32}P]ATP. As seen in Table I, topoisomerase II cleaved *ϕX174* molecules were not labeled by this procedure. In contrast, a linear pBR322 DNA standard and the contaminating linear *ϕX174* (+) strand present in the starting material showed near quantitative phosphorylation. Thus, topoisomerase II appears to be covalently attached to the 5′-termini of cleaved single-stranded DNA.

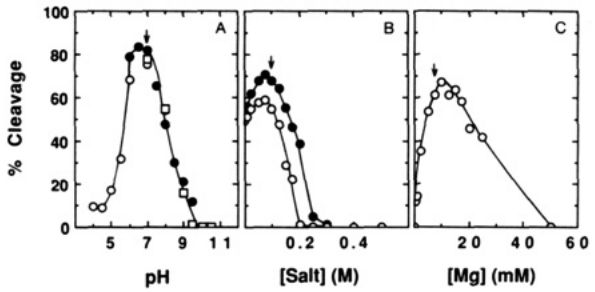

FIGURE 2: Optimal conditions for cleavage of $\phi X174$ (+) strand DNA by topoisomerase II. Assays were carried out as described under Experimental Procedures. Percent cleavage was estimated by the loss of circular $\phi X174$ (+) strand molecules. Panel A: effect of pH on DNA cleavage. Assays were carried out in 10 mM MES (○), Tris (●), or AMPSO (□). Panel B: effect of salt concentration on DNA cleavage. Assays contained either NaCl (○) or KCl (●). Panel C: effect of magnesium concentration on DNA cleavage. In all panels, optimal conditions for cleavage of double-stranded pBR322 DNA by *Drosophila* topoisomerase II are indicated for comparison by the arrow.

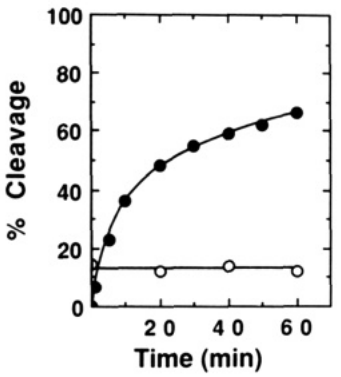

FIGURE 3: Time course for topoisomerase II mediated DNA cleavage. Assays were carried out as described under Experimental Procedures. The cleavage of single-stranded $\phi X174$ DNA (●) and double-stranded pBR322 DNA (○) are shown. The cleavage of $\phi X174$ DNA was estimated by the loss of circular molecules. The cleavage of pBR322 was determined by the increase in linear molecules.

The effects of pH, ionic strength, and magnesium concentration on the cleavage of single-stranded $\phi X174$ DNA by topoisomerase II are shown in Figure 2. Optimal cleavage was observed at pH 6.5–7.0 (panel A) at 50–100 mM salt with either NaCl or KCl (panel B) in the presence of ~10 mM $MgCl_2$ (panel C). These values are similar to those that promote maximal cleavage of double-stranded DNA by the *Drosophila* type II enzyme (as denoted by the arrows in Figure 2) (Osheroff, 1987; Zechiedrich et al., 1989). In addition, as previously shown with double-stranded molecules (Sander & Hsieh, 1983; Osheroff, 1987), cleavage of single-stranded DNA absolutely required the presence of a divalent cation (panel C).

Role of the Protein Denaturant in the Topoisomerase II Mediated DNA Cleavage Assay. As monitored by the in vitro assay, topoisomerase II mediated cleavage of double-stranded DNA is an equilibrium process. Thus, cleavage is time independent (beyond 5 s) (see Figure 3) and can be reversed by the addition of salt (Liu et al., 1983; Osheroff & Zechiedrich, 1987; Osheroff, 1989a; Zechiedrich et al., 1989). In order to observe the cleavage of double-stranded DNA by topoisomerase II, in vitro assays must be terminated by the addition of a strong protein denaturant such as SDS (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff, 1987). Because the enzyme forms covalent bonds with both 5′-termini generated by cleavage, it has been difficult to define the role of the protein denaturant in this process. It is not clear whether SDS traps a steady-state enzyme complex in which the DNA is already cleaved or whether the denaturant induces hydrolysis of the DNA backbone in a precleavage complex (Liu et al., 1983; Wang, 1985; Vosberg, 1985; Osheroff, 1989a). To address this point, it is necessary to determine whether the equilibrium observed with double-stranded substrates is established between DNA cleavage and religation or rather reflects a precleavage process.

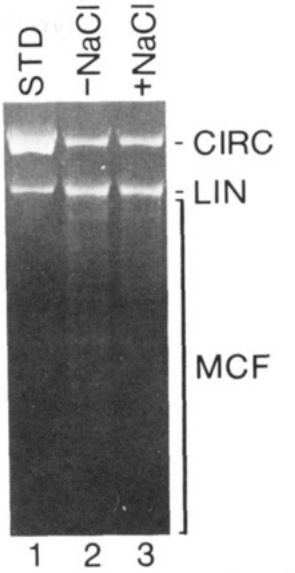

FIGURE 4: Irreversibility of single-stranded $\phi X174$ DNA cleavage mediated by topoisomerase II. Assays were carried out as described under Experimental Procedures. An agarose gel is shown. Lane 1, $\phi X174$ (+) strand DNA standard; lane 2, cleavage carried out for 60 min; lane 3, cleavage carried out for 60 min followed by the addition of 0.5 M NaCl and a further 10-min incubation. The positions of circular, linear, and multiply cleaved $\phi X174$ molecules are as shown in Figure 1.

Single-stranded $\phi X174$ DNA was used to distinguish between these two possibilities. Since topoisomerase II forms a covalent linkage with the single 5′-terminus generated during cleavage (Figure 1 and Table I), the newly formed 3′-OH terminus may be able to diffuse away from the enzyme's active site. Such an event should uncouple the enzyme's normal DNA cleavage/religation cycle. Therefore, if DNA cleavage takes place prior to the addition of SDS, uncoupling this cycle should disrupt the equilibrium observed with double-stranded substrates. In this case, cleavage of single-stranded molecules should be time dependent, should not be salt reversible, and should not require the addition of SDS. In contrast, if SDS induces topoisomerase II to hydrolyze the nucleic acid backbone within a precleavage complex, the in vitro cleavage of single-stranded DNA should still reflect an equilibrium process.

By the three criteria listed above, SDS appears to trap a topoisomerase II complex in which the DNA has already been cleaved. First, cleavage of circular $\phi X174$ (+) strand DNA was time dependent. As seen in Figure 3 (closed circles), levels of cleavage continued to rise for at least 60 min. At early reaction times (≤10 min), products were predominantly unit-length linear molecules. At longer reaction times, subunit-length fragments (which result from multiple cleavage events per $\phi X174$ molecule) became more apparent. No loss of circular $\phi X174$ DNA was observed at 60 min in the absence of enzyme. A time course for the cleavage of double-stranded pBR322 plasmid DNA by topoisomerase II is shown for comparison (Figure 3, open circles).

Second, cleavage of single-stranded $\phi X174$ DNA was not reversed by the addition of salt (Figure 4). In this experiment, DNA cleavage was carried out for 60 min as described under Experimental Procedures. However, prior to the addition of

Table II: Termination of DNA Cleavage[a]

termination method[b]	cleavage (%) 10 min	60 min
SDS (0.5%)	38	54
alkali (0.1 N NaOH)	36	74
heat (70 °C, 2 min)	25	60
EDTA (25 mM)	36	60
proteinase K	58	72

[a] The percent of single-stranded DNA cleavage was estimated by the loss of the circular substrate. These results represent the average of two independent experiments. [b] Following termination, reactions were processed as described under Experimental Procedures. All samples were treated with SDS, EDTA, proteinase K, and heat; however, the termination method listed preceded the other treatments. When alkali was used to terminate reactions, samples were neutralized with 0.1 N HCl prior to further processing.

SDS, NaCl (0.5 M final concentration) was added and the sample was incubated for an additional 10 min at 30 °C (lane 3). No increase in the level of circular *ϕX174* substrate was observed following this salt incubation (compare lanes 2 and 3). The concentration of NaCl employed in this study completely inhibits the enzyme's ability to cleave single-stranded DNA (see Figure 2) and readily reverses topoisomerase II mediated cleavage of double-stranded DNA molecules (Liu et al., 1983; Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989).

Finally, SDS was not required to observe cleavage of single-stranded *ϕX174* DNA by topoisomerase II (Table II). Comparable levels of cleavage were generated at either 10 or 60 min when reactions were terminated by the addition of SDS, alkali, heat, EDTA, or proteinase K. A similar result was found for the bacteriophage T4 enzyme when EDTA was used to terminate the cleavage of *ϕX174* (+) strand DNA (Kreuzer, 1984). Topoisomerase II mediated cleavage of double-stranded DNA is not observed when heat, EDTA, or proteinase K is employed to terminate assays (Sander & Hsieh, 1983; Osheroff & Zechiedrich, 1987).

Regardless of the termination method employed, all *ϕX174* samples were processed with proteinase K prior to analysis by agarose gel electrophoresis (see Table II, footnote *b*). In a parallel series of experiments, reactions were terminated with SDS, alkali, heat, or EDTA, but were not treated with proteinase K (not shown). In all cases, a loss of circular *ϕX174* substrate similar to that shown in Table II was observed. However, in the absence of the proteolytic enzyme, DNA cleavage products were enzyme bound. Thus, topoisomerase II forms a covalent linkage with cleaved single-stranded molecules when any of the above methods are used to terminate reactions.

Generation of Free 3′-OH DNA Termini Following Incubation of Circular ϕX174 (+) Strand Molecules with Topoisomerase II. The data presented above strongly suggest that topoisomerase II mediates DNA cleavage prior to the addition of SDS. If this conclusion is correct, each cleavage event on *ϕX174* (+) strand DNA should generate a single free 3′-OH terminus.

To provide direct evidence for such an enzyme-generated or terminus, single-stranded circular *ϕX174* DNA was incubated for 10 or 60 min with topoisomerase II and then further incubated with terminal deoxynucleotidyltransferase and [α-^{32}P]ddATP. Samples were digested with proteinase K, ethanol precipitated, and subjected to electrophoresis on an agarose gel. DNA cleavage products were visualized by autoradiography. As seen in Figure 5, both unit-length and smaller linear *ϕX174* fragments generated by topoisomerase II in the absence of a protein denaturant contained free 3′-OH termini that were

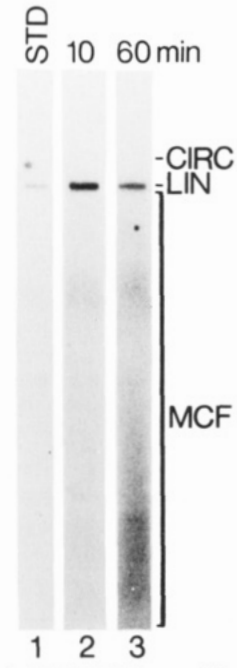

FIGURE 5: Labeling the 3′-OH DNA termini generated by incubation of circular *ϕX174* (+) strand molecules with topoisomerase II. Assays were carried out as described under Experimental Procedures. An autoradiogram is shown. Lane 1, *ϕX174* (+) strand DNA standard; lanes 2 and 3, cleavage reactions carried out for 10 or 60 min, respectively, prior to labeling with terminal deoxynucleotidyltransferase. The positions of circular, linear, and multiply cleaved *ϕX174* DNA molecules are as shown in Figure 1.

labeled by terminal deoxynucleotidyltransferase. The small amount of radioactive label incorporated into the *ϕX174* standard (lane 1) reflects the linear contaminant present in the original DNA substrate (see Figure 1). After a 10-min incubation with topoisomerase II, most of the incorporated [α-^{32}P]ddAMP was found in unit-length linear molecules (lane 2). At longer reaction times (such as 60 min), considerable amounts of label were observed in sub-unit-length DNA fragments (lane 3). As discussed in the previous section, these findings correlate well with the distribution of DNA cleavage products at these two incubation times.

All of the 3′-OH DNA termini produced over the course of the reaction were generated by topoisomerase II cleavage. In the absence of the enzyme, radioactive incorporation remained at the background level seen in lane 1 (Figure 5), even at 60 min. Furthermore, all of the labeled cleavage products were covalently attached to topoisomerase II. When reaction mixtures were not treated with proteinase K prior to electrophoresis, labeled unit-length linear (excluding the original contaminant) and smaller *ϕX174* DNA fragments remained at the origin of the gel (not shown).

In order to quantitate the incorporation of [α-^{32}P]ddAMP onto topoisomerase II generated 3′-OH DNA termini, samples were analyzed by filter binding and scintillation counting. A time course for the enzyme-mediated cleavage of circular *ϕX174* (+) strand DNA is shown in Figure 6 (open circles). Data points were corrected for the linear contaminant present in the initial *ϕX174* DNA substrate as well as the topoisomerase II mediated cleavage that takes place during the incubation with terminal deoxynucleotidyltransferase.

At all time points examined, the incorporation of [α-^{32}P]-ddAMP (closed circles) paralleled the level of DNA cleavage observed (as approximated by the loss of circular *ϕX174* substrate). Unfortunately, it is impossible to quantitate the actual number of cleavage events that take place in this ex-

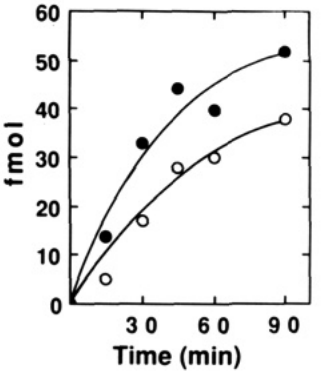

FIGURE 6: Time course for the generation of 3′-OH DNA termini in *ϕX174* (+) strand DNA by topoisomerase II. Assays were carried out as described under Experimental Procedures. The incorporation of [α-^{32}P]ddAMP onto newly generated 3′-OH termini (●) was quantitated by a filter binding assay. The cleavage of single-stranded *ϕX174* DNA (○) was estimated by the loss of circular substrate. Initial reaction mixtures contained 100 fmol of *ϕX174* (+) strand DNA.

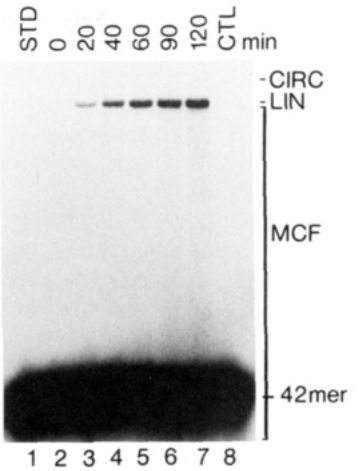

FIGURE 7: Time course for the topoisomerase II mediated intermolecular ligation of cleaved *ϕX174* (+) strand DNA onto a radioactively labeled double-stranded oligonucleotide. Assays were carried out as described under Experimental Procedures. An autoradiogram is shown. Lane 1, *ϕX174* (+) strand DNA and oligonucleotide (blunt-ended 42-mer) standards; lanes 2–7, *ϕX174* (+) strand DNA incubated for 5 min with topoisomerase II followed by incubation with the double-stranded oligonucleotide for 0, 20, 40, 60, 90, or 120 min, respectively; lane 8, control (CTL) reaction equivalent to lane 5 except that topoisomerase II was not included in the reaction mixture. The positions of circular, linear, and multiply cleaved *ϕX174* molecules are as shown in Figure 1. The electrophoretic mobility of the labeled 42-mer is also shown.

periment because some of the input *ϕX174* DNA is cleaved by more than one enzyme molecule (see Figures 1 and 5). Although the method employed underestimates the actual number of cleavage events, it is clear that the majority of linear *ϕX174* DNA cleavage products are labeled by terminal deoxynucleotidyltransferase. Indeed, following a 90-min incubation with topoisomerase II, over half of the input DNA substrate was labeled with [α-^{32}P]ddAMP.

These results confirm that topoisomerase II generates a 3′-OH terminus in single-stranded *ϕX174* DNA in the absence of any protein denaturant. Therefore, enzyme-mediated DNA cleavage must take place prior to the addition of SDS in the in vitro assay.

Intermolecular DNA Ligation Mediated by Topoisomerase II. In order to demonstrate that topoisomerase II in the enzyme-cleaved *ϕX174* (+) strand complex is kinetically competent, the ability of topoisomerase II to perform intermolecular ligation of the cleaved DNA was determined. To this end, topoisomerase II was incubated for 5 min with single-stranded circular *ϕX174* DNA followed by a further incubation with a 5-fold molar excess (with respect to *ϕX174*) of a radioactively labeled double-stranded 42-bp oligonucleotide. A time course for the oligonucleotide incubation is shown in Figure 7.

Topoisomerase II ligated *ϕX174* (+) strand DNA to the 42-mer in a time-dependent fashion. Only cleaved *ϕX174* molecules were ligated to the oligonucleotide, as no radioactive label was observed in the circular DNA band. Intermolecular ligation was inhibited proportionally when nonphosphorylated competitor 42-mer was added to incubation mixtures. This indicates that the reaction requires the oligonucleotide but not the presence of its phosphorylated 5′-terminus. Finally, intermolecular ligation was observed when labeled 42-mer was replaced in assays by other radioactive oligonucleotides (not shown). This finding demonstrates that the reaction is not exclusive to the 42-mer employed in the present study.

To quantitate levels of intermolecular ligation, bands were excised from the agarose gel and their radioactivity was measured by liquid scintillation counting. Following a 60-min incubation (lane 5), approximately 2.5% of the *ϕX174* unit-length linear molecules were covalently attached to the 42-mer. Similar levels of the multiply cleaved *ϕX174* linears were also ligated to the labeled oligonucleotide.

The efficiency of intermolecular ligation is low relative to that observed for the enzyme's intramolecular religation of cleaved double-stranded DNA (Osheroff & Zechiedrich, 1987; Osheroff, 1989b; Robinson & Osheroff, 1990). This is consistent with the fact that the primary physiological ligation reaction of topoisomerase II is intramolecular in nature (Wang, 1985; Vosberg, 1985; Osheroff, 1989a). It should be noted, however, that levels of topoisomerase II mediated intermolecular ligation observed in the present study are 5–10-fold higher than those previously reported for the eukaryotic type I enzyme (Been & Champoux, 1981).

Several lines of evidence indicate that the incorporation of radioactivity into cleaved *ϕX174* (+) strand DNA results from an intrinsic topoisomerase II mediated ligation activity. First, active topoisomerase II was required for the reaction. No incorporation of label was observed if topoisomerase II was left out of reaction mixtures (Figure 7, lane 8) or if the enzyme was heat denatured following cleavage of *ϕX174* but before the addition of the radioactive 42-mer. Moreover, levels of incorporation were proportional to the levels of topoisomerase II employed in the assay. Second, similar levels of intermolecular ligation were observed when three independent preparations of *Drosophila* topoisomerase II were used (two from tissue culture cells and one from embryos). Third, the incorporation of label was not due to a possible contaminating kinase activity in the topoisomerase II preparation (Sander et al., 1984). No label was transferred onto cleaved *ϕX174* DNA molecules when the radioactive 42-mer was replaced by a mixture of nonradioactive oligonucleotide and [γ-^{32}P]ATP. Fourth, it is extremely unlikely that label incorporation was due to a contamination with DNA ligase. *Drosophila* DNA ligase elutes from hydroxylapatite and phosphocellulose (the chromatographic media used for topoisomerase II purification) at one-half to one-third the salt concentrations necessary for the elution of topoisomerase II and has a sedimentation coefficient on glycerol gradients (also used in the purification) which is less than half that of the topoisomerase (Shelton et al., 1983; Rabin et al., 1986). Furthermore, *Drosophila* DNA ligase requires 0.5 mM ATP for optimal activity (Rabin et al., 1986), and the present intermolecular ligation [like the

intramolecular religation reaction of topoisomerase II (Zechiedrich et al., 1989)] was observed in the absence of ATP.[2] Fifth, intermolecular ligation assays were inhibited over 80% by the addition of anti-*Drosophila* topoisomerase II antiserum. Previous studies have shown this antiserum to be highly specific for topoisomerase II, even in whole cell lysates (Berrios et al., 1985; Ackerman et al., 1988).

The ability to carry out intermolecular ligation of cleaved *φX174* (+) strand molecules demonstrates that topoisomerase II in the covalent enzyme–cleaved DNA complex is kinetically competent. Thus, it is likely that this complex is representative of the cleavage intermediate that is formed during the course of the enzyme's physiological DNA strand passage reaction.

Discussion

Circular single-stranded *φX174* DNA has been used as a model substrate to examine the DNA cleavage/religation reaction of eukaryotic topoisomerase II. On the basis of the following criteria, topoisomerase II cleaves DNA in vitro prior to the addition of a protein denaturant. First, cleavage of single-stranded DNA is time dependent. Second, reactions are not salt reversible. Third, similar levels of cleavage are observed when reactions are terminated by a variety of methods. Fourth, incubation of circular *φX174* (+) strands with topoisomerase II generates a concentration of 3′-OH termini that is commensurate with the level of DNA cleavage observed. The findings of this study are not unique to the *φX174* molecule. Similar results were obtained when circular single-stranded M13 DNA was employed as substrate (not shown).

As determined by its ability to ligate cleaved *φX174* (+) strand DNA to a double-stranded oligonucleotide, topoisomerase II in the covalent enzyme–cleaved DNA complex is kinetically competent. Thus, SDS allows DNA cleavage to be visualized in vitro by trapping an active topoisomerase II–cleaved DNA complex. As discussed earlier, all available evidence is consistent with the belief that the complex formed with single-stranded molecules is representative of the cleavage intermediate formed during the enzyme's physiological double-stranded DNA passage reaction. However, the possibility that topoisomerase II cleaves single-stranded and double-stranded nucleic acid substrates by different mechanisms cannot be ruled out at the present time.

The topoisomerase II–cleaved DNA complex has been referred to as the "cleavable complex" (Nelson et al., 1984; D'Arpa & Liu, 1989). This operational definition was used to denote that enzyme–nucleic acid complex which yielded topoisomerase II linked cleaved DNA upon treatment with a protein denaturant (Nelson et al., 1984). The term cleavable complex was originally introduced because it was not clear whether SDS induced cleavage to take place within a precleavage enzyme–DNA complex or whether it in fact trapped a cleavage complex in which the DNA backbone was already hydrolyzed (Nelson et al., 1984; Wang, 1985; Vosberg, 1985; Osheroff, 1989a). Since the results of the present study indicate that SDS acts by the latter mechanism, it is suggested that the term cleavable complex be replaced by the term "cleavage complex" to avoid possible mechanistic ambiguities.

A previous investigation of the interaction between bacteriophage T4 topoisomerase II and *φX174* (+) strand molecules mapped several strong sites of enzyme-mediated cleavage to DNA regions with potential for secondary structure (Kreuzer, 1984). These sites were found in single-stranded regions that were adjacent to the base of hairpin loops. This suggests that topoisomerase II may require at least partially duplex DNA structures for nucleic acid recognition and/or cleavage. Whether cleavage by the *Drosophila* type II enzyme is localized primarily to single-stranded regions on *φX174* (+) strand DNA or to regions with potential for secondary structure is not known. If the predominant cleavage sites map at or near regions with duplex character, this would reinforce the use of *φX174* (+) strand DNA as a model substrate for studying the enzyme's physiological activity.

Finally, it has been proposed that topoisomerase II plays a role in mediating or regulating DNA recombination in eukaryotic systems (Bae et al., 1988; Christman et al., 1988; Kim & Wang, 1989a; Sperry et al., 1989; Dillehay et al., 1989; Rose et al., 1990). This proposal is supported by the finding that topoisomerase II targeted antineoplastic drugs which act by stabilizing the enzyme's DNA cleavage intermediate increase chromosomal translocations, mutations, and sister chromatid exchange in treated cells (Pommier et al., 1985; Lim et al., 1986; Renault et al., 1987; DeVore et al., 1989; Lönn et al., 1989; Andersson & Kihlman, 1989; Dillehay et al., 1989). Elucidating the mechanism by which topoisomerase II covalently links nonhomologous nucleic acids may offer insight into how the enzyme can promote illegitimate recombination events. The intermolecular ligation assay presented in this study provides an efficient in vitro model in which to study the mechanism of this potentially important process.

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[2] Even if the gel filtration and ethanol precipitation procedures were completely ineffective at removing unincorporated [γ-^{32}P]ATP from the oligonucleotide phosphorylation reactions, the carry-over into the intermolecular ligation assay mixtures would be less than 1 nM.

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