Double-Stranded DNA Cleavage/Religation Reaction of Eukaryotic Topoisomerase II: Evidence for a Nicked DNA Intermediate†

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ABSTRACT: The DNA cleavage reaction of eukaryotic topoisomerase II produces nicked DNA along with linear nucleic acid products. Therefore, relationships between the enzyme's DNA nicking and double-stranded cleavage reactions were determined. This was accomplished by altering the pH at which assays were performed. At pH 5.0, *Drosophila melanogaster* topoisomerase II generated predominantly (>90%) single-stranded breaks in duplex DNA. With increasing pH, less single-stranded and more double-stranded cleavage was observed, regardless of the buffer or the divalent cation employed. As has been shown for double-stranded DNA cleavage, topoisomerase II was covalently bound to nicked DNA products, and enzyme-mediated single-stranded cleavage was salt reversible. Moreover, sites of single-stranded DNA breaks were identical with those mapped for double-stranded breaks. To further characterize the enzyme's cleavage mechanism, electron microscopy studies were performed. These experiments revealed that separate polypeptide chains were complexed with both ends of linear DNA molecules generated during cleavage reactions. Finally, by use of a novel religation assay [Osheroff, N., & Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303–4309], it was shown that nicked DNA is an obligatory kinetic intermediate in the topoisomerase II mediated reunion of double-stranded breaks. Under the conditions employed, the apparent first-order rate constant for the religation of the first break was approximately 6-fold faster than that for the religation of the second break. The above results indicate that topoisomerase II carries out double-stranded DNA cleavage/religation by making two sequential single-stranded breaks in the nucleic acid backbone, each of which is mediated by a separate subunit of the homodimeric enzyme.

The topological state of DNA plays an important role in determining how its genetic information is regenerated, expressed, and recombined in the cell (Wang, 1985; Vosberg, 1985). In vivo, the topology of DNA is modulated by two ubiquitous enzymes, type I and type II topoisomerases (Wang, 1985; Vosberg, 1985). Topoisomerase II acts by introducing a transient double-stranded break in duplex DNA, passing a second DNA helix through the break, and resealing the cleaved DNA (Wang, 1985; Vosberg, 1985; Osheroff, 1989). The enzyme is essential for the viability of the eukaryotic cell (Goto & Wang, 1984; DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985) and functions in DNA replication (Sundin & Varshavsky, 1981; Nojuchi et al., 1983; Jazwinski & Edelman, 1984; Weaver et al., 1985; Nelson et al., 1986; Snapka, 1986; Yang et al., 1987; Brill et al., 1987) and transcription (Brill et al., 1987; Glikin & Blangy, 1986; Rowe et al., 1986), as well as chromosome condensation (Newport, 1987; Newport & Spann, 1987; Uemura et al., 1987) and segregation (DiNardo et al., 1984; Uemura & Yanagida, 1984; Holm et al., 1985; Uemura et al., 1987; Uemura & Yanagida, 1986). In addition, the type II enzyme plays structural roles in maintaining the organization of the mitotic chromosome scaffold (Earnshaw & Heck, 1985; Earnshaw et al., 1985; Gasser & Laemmli, 1986; Gasser et al., 1986) and the interphase nuclear matrix (Berrios et al., 1985; Cockerill & Garrard, 1986). Beyond its physiological functions, topoisomerase II is the cellular target for several classes of clinically relevant chemotherapeutic drugs (Zwelling, 1985; Glisson & Ross, 1987).

The DNA cleavage reaction of topoisomerase II has been the subject of intense interest. It has been employed to define the enzyme's sites of interaction on DNA and chromatin (Yang et al., 1985; Sander & Hsieh, 1983, 1985; Udvardy et al., 1985, 1986; Rowe et al., 1986). Furthermore, the antineoplastic activities of many chemotherapeutic agents correlate with their abilities to stabilize topoisomerase II–DNA cleavage complexes (Zwelling, 1985; Glisson & Ross, 1987). Despite the importance of the enzyme's DNA cleavage reaction, the detailed kinetic pathway of this process has yet to be described.

To observe the topoisomerase II–DNA cleavage complex, the enzyme and DNA are mixed and allowed to establish a cleavage/religation equilibrium. DNA cleavage products are obtained by adding a rapidly acting protein denaturant, such as sodium dodecyl sulfate (SDS),[1] to reaction mixtures (Sander & Hsieh, 1983; Liu et al., 1983). Two alternatives have been proposed for the role of SDS in the DNA cleavage reaction (Wang, 1985; Vosberg, 1985; Osheroff, 1989): (1) SDS induces enzyme-mediated DNA cleavage to take place within an existing precleavage (i.e., "cleavable") topoisomerase II–DNA complex or (2) SDS rapidly denatures and thereby traps a complex in which the nucleic acid has already been cleaved

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[1] Abbreviations: SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid); Bistris, [bis(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mops, 3-(*N*-morpholino)propanesulfonic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid); Ampso, 3-[*N*-(α,α-dimethylhydroxyethyl)amino]-2-hydroxypropanesulfonic acid.

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by the enzyme. On the basis of mechanistic comparisons with eukaryotic topoisomerase I (Been & Champoux, 1980, 1981; Prell & Vosberg, 1980; Halligan et al., 1982) the latter interpretation is probably correct. This conclusion is supported by a recent study (Gale & Osheroff, 1989) which indicated that the 3′-terminus of DNA within the enzyme–DNA cleavage complex is available for labeling prior to the addition of SDS.

Invariably, topoisomerase II mediated DNA cleavage produces not only double-stranded breaks in the nucleic acid backbone but single-stranded breaks (i.e., nicks) as well (Nelson et al., 1984; Tewey et al., 1984; Pommier et al., 1985; Liu et al., 1983; Osheroff & Zechiedrich, 1987; Muller et al., 1988). The substitution of divalent calcium for magnesium in reaction mixtures exacerbates this DNA nicking (Osheroff & Zechiedrich, 1987). Moreover, the presence of antineoplastic agents increases both double- and single-stranded DNA cleavage (Nelson et al., 1984; Tewey et al., 1984; Pommier et al., 1984, 1985; Yang et al., 1985). While the physiological implications of enzyme-mediated DNA nicking are unknown, it has been suggested that single-stranded breaks are intermediates in the double-stranded DNA cleavage reaction of topoisomerase II (Osheroff & Zechiedrich, 1987; Muller et al., 1988).

To further characterize the kinetic mechanism of *Drosophila melanogaster* topoisomerase II, the role of DNA nicks in the enzyme's cleavage/religation reaction was determined. To this end, three approaches were employed. First, conditions were established that promoted high levels of enzyme-mediated single-stranded cleavage in duplex DNA. Second, products of the double-stranded cleavage reaction were examined by electron microscopy. Third, the kinetic pathway of the enzyme's DNA religation reaction was delineated. The results of this study strongly suggest that topoisomerase II cleaves/religates DNA by making two coordinated single-stranded nicks in the nucleic acid backbone. Moreover, each single-stranded break is mediated by a separate subunit of the homodimeric enzyme. Finally, at least during religation, double-stranded DNA breaks are rejoined in a sequential fashion.

A preliminary account of this work has appeared (Zechiedrich et al., 1988).

Experimental Procedures

DNA topoisomerase II was purified from the nuclei of *D. melanogaster* Kc tissue culture cells by the procedure of Shelton et al. (1983). The isolated enzyme was stored in 15 mM sodium phosphate, pH 7.1, 700 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 35% glycerol and placed in liquid nitrogen until use. Once thawed, it was kept as a liquid at −20 °C. Negatively supercoiled bacterial plasmid pBR322 (Bolivar et al., 1977) DNA was isolated from *Escherichia coli* DH1 (Hanahan, 1983) by a Triton X-100 lysis procedure followed by double banding in cesium chloride–ethidium bromide gradients (Maniatis et al., 1982). Analytical reagent grade $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$, were obtained from Fisher; ethidium bromide, Tris, protein molecular weight markers, and goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate were from Sigma; SDS and proteinase K were from E. Merck Biochemicals; imidazole, Mes, Bistris, Pipes, Mops, Hepes, and Ampso were from Research Organics Inc. All other chemicals were of analytical reagent grade.

Cleavage of DNA by Topoisomerase II. Unless stated otherwise, rections employed 75 nM topoisomerase II and 5 nM (0.3 μg) negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer (10 mM imidazole, pH 5.0–7.5, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 2.5% glycerol) that contained 5 mM $MgCl_2$ or $CaCl_2$.[2] The pH of completed reaction mixtures was confirmed by using a Lazar Research Laboratories micro pH electrode (Model PHR-146). Samples were incubated at 30 °C for 6 min. DNA cleavage products were trapped by the addition of 2 μL of 10% SDS. One microliter of 250 mM EDTA and 2 μL of 0.8 mg/mL proteinase K were added, and mixtures were incubated at 45 °C for 30 min to digest the topoisomerase II. Products were mixed with 3 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris-HCl, pH 7.9), heated at 75 °C for 2 min, and subjected to electrophoresis in 1.0% agarose (MCB) gels in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm until the bromophenol blue had traveled approximately 12.5 cm (3–4 h). Gels were stained for 35 min in an aqueous solution of ethidium bromide (1 μg/mL). DNA bands were visualized by transillumination with ultraviolet light (300 nm) and photographed through Kodak 23A and 12 filters with Polaroid 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.

Mapping of Topoisomerase II Cleavage Sites. A strong recognition site for topoisomerase II mediated double-stranded DNA cleavage has been located close to the origin of replication in the rDNA promoter region of *Tetrahymena thermophila*. The properties of this recognition site are described in the following paper (Andersen et al., 1989). A cloned 365-bp *Hin*dIII–*Bam*HI fragment that encompassed this site was labeled at both protruding 5′-ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (Maniatis et al., 1982). DNA labeled only on the 5′-end of the noncoding strand was obtained by digesting this fragment with the restriction endonuclease *Xba*I. The resulting 276-bp single-end-labeled *Hin*dIII–*Xba*I fragment was employed for mapping experiments. The labeled rDNA (75 nM) was incubated with topoisomerase II (150 nM) at 30 °C for 10 min in 20 μL of 10 mM Tris-HCl, pH 7.5, or 10 mM imidazole–HCl, pH 5.5, 5 mM $CaCl_2$ or 5 mM $MgCl_2$, 25 mM NaCl, and 0.1 mM EDTA. The reaction was terminated by the addition of 1% SDS (final concentration). EDTA (10 mM final concentration) and proteinase K (0.3 mg/mL final concentration) were added, and mixtures were digested at 37 °C for 1 h. Samples were mixed with an equal volume of deionized formamide, 0.05% bromophenol blue, 0.03% xylene cyanol FF, and 5 mM EDTA, pH 8.5, and subjected to electrophoresis on a denaturing polyacrylamide gel (6% acrylamide, 0.3% bis(acrylamide), and 8 M urea) for 3 h in 89 mM Tris–borate, pH 8.3, and 2 mM EDTA. Reaction products were visualized by autoradiography using Kodak XAR film and a Du Pont Lightning Plus screen.

Religation of Cleaved DNA by Topoisomerase II. The religation assay of Osheroff and Zechiedrich (1987) was employed. Topoisomerase II (75 nM) and 5 nM negatively supercoiled pBR322 DNA were incubated for 6 min at 30 °C in 20 μL of cleavage buffer that contained 5 mM $CaCl_2$. Kinetically competent covalent topoisomerase II–DNA complexes were trapped by the addition of 2 μL of 100 mM EDTA. NaCl (0.8 μL of a 5 M solution) was added to prevent recleavage of the DNA during the religation reaction (Liu et

[2] Under optimal assay conditions, the DNA cleavage/religation equilibrium of topoisomerase II lies heavily toward religation (Osheroff, 1989). Thus, greater than stoichiometric enzyme:DNA ratios were employed.

Table I: Effect of Various Buffers on the Ability of Topoisomerase II To Generate Single-Stranded (SS) or Double-Stranded (DS) Breaks in Duplex DNA[a]

		pH 5.0		pH 5.5		pH 6.5		pH 7.5	
buffer	pK_a	Ca^{2+} (SS:DS)	Mg^{2+} (SS:DS)	Ca^{2+} (SS:DS)	Mg^{2+} (SS:DS)	Ca^{2+} (SS:DS)	Mg^{2+} (SS:DS)	Ca^{2+} (SS:DS)	Mg^{2+} (SS:DS)
Mes	5.96	99:1	98:2	97:3	97:3	71:29	69:31	58:42	46:54
Bistris	6.36	99:1	99:1	99:1	97:3	80:20	73:27	64:36	65:35
Pipes	6.66	99:1	97:3	96:4	96:4	68:32	69:31	56:44	60:40
imidazole	6.75	91:9	92:8	91:9	90:10	69:31	64:36	56:44	61:39
Mops	7.01	96:4	98:2	96:4	94:6	70:30	78:22	55:45	55:45
Hepes	7.31					69:31	75:25	60:40	62:38
Tris	7.77					64:36	81:19	60:40	53:47
Ampso	9.1							62:38	56:44

[a] DNA cleavage reactions were carried out in the presence of calcium or magnesium in the indicated buffers as described under Experimental Procedures. Total DNA breaks (i.e., single-stranded plus double-stranded) were normalized to 100. The pK_a values for the buffers employed are shown.

al., 1983; Osheroff & Zechiedrich, 1987). Samples were placed on ice to slow reaction rates, and religation was initiated by the addition of cold $MgCl_2$ (7.5 mM final concentration). SDS (2 μL of a 10% solution) was added to terminate religation at various time points up to 30 s. Samples were digested with proteinase K and subjected to electrophoresis as described above.

Glycerol Gradient Centrifugation. Gradients (11.25 mL) ranging from 15% to 40% glycerol in 15 mM sodium phosphate buffer, pH 5.0 or 7.5, 0.1 mM EDTA, and 700 mM NaCl were employed (Shelton et al., 1983). Topoisomerase II (11 μg), along with catalase, fibrinogen, ovalbumin, and cytochrome *c* standards, was loaded in a total of 160 μL. Gradients were centrifuged at 39 000 rpm at 4 °C for 65 h in a Beckman SW-41 rotor. Protein standards were monitored spectrophotometrically at A_{280nm} or A_{410nm} and topoisomerase II was monitored either by a DNA relaxation assay (Osheroff et al., 1983) or by the immunoblot procedure described below.

Immunoblot Analysis. Samples (100 μL) across glycerol gradients were applied to a presoaked nitrocellulose membrane under vacuum by use of a Schleicher and Schuell Miniblot II slot blot apparatus according to the procedure of Blake et al. (1984). The nitrocellulose was incubated in Tris-buffered saline (TBS) (20 mM Tris, pH 7.4, 500 mM NaCl) containing 3% gelatin and then washed twice for 5 min in TBS containing 0.05% Tween 20. Rabbit anti-*Drosophila* topoisomerase II antiserum (Shelton et al., 1983; Berrios et al., 1985) was applied at a dilution of 1:250 for 1 h. The nitrocellulose was washed twice with TBS and then incubated for 1 h with goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate. Following two 5-min washes with TBS, immunoblots were developed in a mixture of nitro blue tetrazolium (30 mg/mL) and 5-bromo-4-chloroindolyl phosphate (15 mg/mL).

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were prepared and subjected to electrophoresis according to the procedure of Laemmli (1970). The stacking gel was 4.5% and the running gel was 7% acrylamide. Proteins were visualized with Coomassie Brilliant Blue R-250.

Electron Microscopy. Topoisomerase II–DNA complexes were generated at pH 7.5 in the presence of 5 mM $CaCl_2$ as described above except that the concentration of enzyme was reduced to 25 nM. Cleavage products were trapped by the addition of EDTA (5 mM final concentration) (Osheroff & Zechiedrich, 1987). SDS (0.025% final concentration) was added and samples were heated at 60 °C for 2 min to dissociate enzyme subunits. $MgCl_2$ was added to a final concentration of 5 mM to facilitate nucleic acid binding to grids. Mixtures were diluted ~15-fold in buffer containing 50 mM KCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.9, and 0.2 mM dithiothreitol. Samples were then applied to polylysine-coated grids prepared as described by Williams (1977). The grids were rotary shadowed at a 6° angle with an 80:20 platinum:palladium mix and analyzed with a Hitachi H-800 electron microscope.

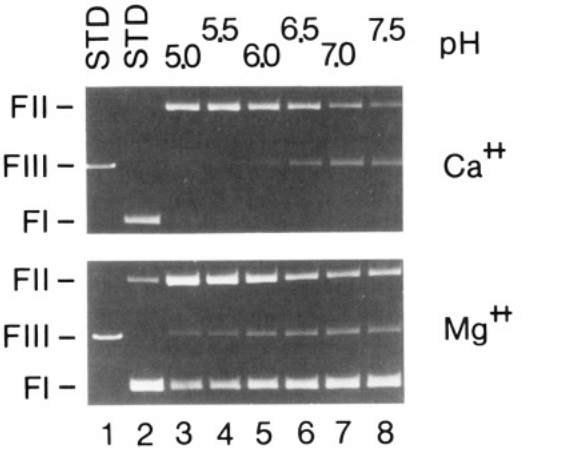

FIGURE 1: pH titration of the DNA cleavage reaction of *D. melanogaster* topoisomerase II. Assays contained 5 nM negatively supercoiled pBR322 DNA and 100 nM enzyme and were carried out at the indicated pH in the presence of either calcium (top) or magnesium ions (bottom) as described under Experimental Procedures. The positions of form I (FI), fully supercoiled DNA; form II (FII), nicked circular plasmid molecules; and form III (FIII), linear molecules, are shown. (Lane 1) Linear DNA standard; (lane 2) pH 5.0 minus topoisomerase II; (lanes 3–8) enzyme-mediated DNA cleavage at pH 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5, respectively.

Results

Effect of pH on the DNA Cleavage Reaction of Topoisomerase II. To more fully characterize the DNA cleavage reaction of *Drosophila* topoisomerase II, the effect of pH on enzyme-mediated DNA cleavage was examined. At pH 7.5, high levels of double- and single-stranded DNA cleavage were observed (Figure 1). As the pH of the reaction was lowered, levels of double-stranded DNA cleavage decreased while DNA nicking increased. At pH 5.0, >90% of the observed cleavage was single-stranded when either divalent magnesium or calcium ions were used to promote DNA cleavage (Figure 1; Table I). Similar results were obtained with the type II topoisomerase from calf thymus (not shown).

The effect of pH was independent of the buffer employed. Using buffers with pK_a values varying over 3 pH units, ratios of single- to double-stranded DNA cleavage were similar at any given pH (Table I). In the absence of topoisomerase II, pH (over the range 5.0–7.5) did not alter the percent of nicked DNA in the negatively supercoiled plasmid standards under the assay conditions employed.

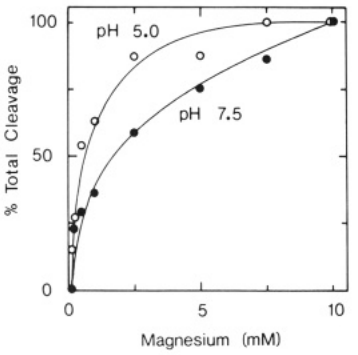

FIGURE 2: Magnesium titration of the topoisomerase II mediated DNA cleavage reaction. Assays were carried out at pH 5.0 (○) or pH 7.5 (●) as described under Experimental Procedures. Total DNA cleavage represents the sum of double- and single-stranded cleavage. Maximal values were normalized to 100%.

The ability of topoisomerase II to utilize magnesium ions at low pH was determined by examining the magnesium-dependence of DNA cleavage at pH 5.0 and 7.5. As shown in Figure 2, the switch from double- to single-stranded DNA cleavage at lowered pH was not due to a decrease in the system's affinity for magnesium, since the total amount of DNA cleavage at any given magnesium concentration was at least as great at pH 5.0 as it was at pH 7.5.

Exposure to pH 5.0 did not appear to cause irreversible changes in the enzyme. Topoisomerase II that was incubated at pH 5.0 for as long as 65 h was still able to mediate high levels of DNA nicking (at pH 5.0), double-stranded cleavage (at pH 7.5), and ATP/Mg^{2+}-dependent DNA relaxation (at pH 7.5). Furthermore, as determined by polyacrylamide gel electrophoresis, incubation under assay conditions (pH 5.0, for 6 min at 30 °C) did not lead to degradation of topoisomerase II (not shown). Finally, the sedimentation coefficient of *Drosophila* topoisomerase II (9.2 S) (Shelton et al., 1983) was the same at either pH 5.0 or 7.5 (not shown). Therefore, acidic pH did not dissociate the subunits of the homodimeric enzyme.

Covalent Attachment of Topoisomerase II to Nicked DNA. A hallmark of the topoisomerase II mediated double-stranded DNA cleavage reaction is the covalent attachment of the enzyme to its linear DNA product (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987). Nicked DNA, generated by single-stranded cleavage at pH 5.0, was also covalently bound to topoisomerase II. As shown in Figure 3, even after treatment with 1% SDS (final concentration) at 75 °C, both linear (pH 7.5) and nicked products (pH 5.0) had to be digested with proteinase K to release cleaved DNA from the enzyme.

Salt Reversibility of the DNA Nicking Reaction. The double-stranded DNA cleavage reaction of topoisomerase II is readily reversed by the addition of salt when either magnesium (Liu et al., 1983) or calcium (Osheroff & Zechiedrich, 1987) is employed as the divalent cation. When pH 5.0 mixtures containing *Drosophila* topoisomerase II and negatively supercoiled pBR322 plasmid DNA were incubated at 30 °C in 100 mM salt, treated with increasing amounts of sodium chloride, and allowed to reincubate prior to the addition of SDS, nicked DNA circles were converted back to supercoiled molecules (Figure 4); 50% reversal occurred at a total salt concentration of ~420 mM when either magnesium or calcium was used as the divalent cation.

Nucleotide Specificity of Topoisomerase II Mediated DNA Nicking. A strong recognition site for topoisomerase II mediated double-stranded DNA cleavage has been located close

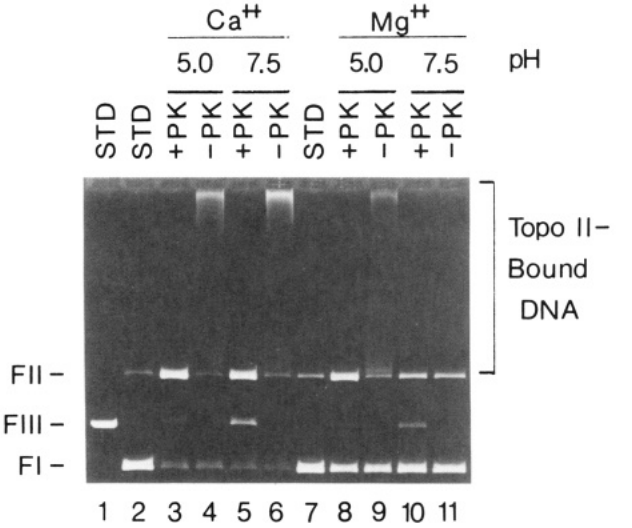

FIGURE 3: Covalent nature of topoisomerase II–nicked DNA complexes. Assays were carried out in the presence of calcium (lanes 3–6) or magnesium (lanes 8–11) as described under Experimental Procedures. The positions of FI, FII, and FIII are as in Figure 1. The position of DNA bound to topoisomerase II is also shown. (Lane 1) Linear DNA standard; (lanes 2 and 7) negatively supercoiled standards; (lanes 3 and 8) assays were at pH 5.0, and products were digested with proteinase K (PK); (lanes 4 and 9) assays were at pH 5.0 with no proteinase K digestion; (lanes 5 and 10) assays were at pH 7.5 with proteinase K; (lanes 6 and 11) assays were at pH 7.5 with no proteinase K.

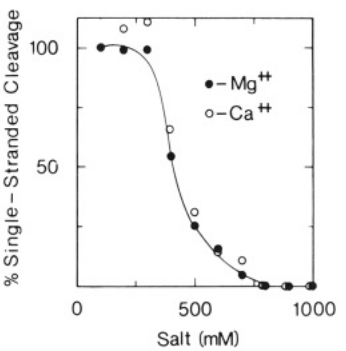

FIGURE 4: Salt reversal of topoisomerase II mediated DNA nicking. Initial enzyme–DNA incubations were in 100 mM salt at pH 5.0 in the presence of magnesium (○) or calcium (●) as described under Experimental Procedures. Salt levels were raised by the addition of concentrated NaCl solutions, and mixtures were allowed to reincubate at 30 °C for 3 min prior to termination with SDS. Initial values of single-stranded DNA cleavage were normalized to 100%.

to the origin of replication in the rDNA promoter region of *T. thermophila* (Andersen et al., 1989). DNA cleavage experiments employed a cloned 276-bp fragment of double-stranded DNA that spanned this recognition site. To map sites of DNA nicking by *Drosophila* topoisomerase II, this double-stranded fragment was radiolabeled at the 5′-terminus of the noncoding rDNA strand and reacted with the type II enzyme (Figure 5). Under conditions that generated predominantly single-stranded (lanes 1 and 3) or double-sstranded (lanes 2 and 4) nucleic acid breaks, sites of DNA scission were identical in the presence of either magnesium or calcium. The DNA cleavage products generated in two independent experiments were quantitated by scanning densitometry. In all cases, greater than 92% of the total observed DNA cleavage took place at the site indicated in Figure 5. Similar results were obtained with calf thymus topoisomerase II (not shown). No cleavage was detected in the absence of enzyme.

Electron Microscopy of Topoisomerase–DNA Reaction Products. When eukaryotic topoisomerase II creates dou-

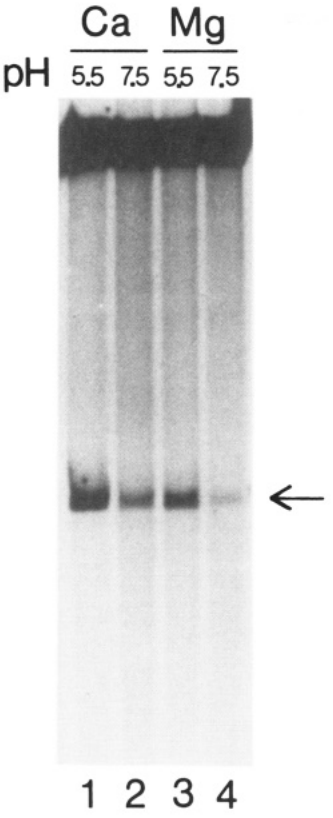

FIGURE 5: Nucleotide specificity of enzyme-mediated DNA nicking. A 276-bp linear fragment of *T. thermophilia* rDNA containing a strong topoisomerase II cleavage site was employed. The DNA was labeled with [^{32}P]phosphate at the 5′-terminus of the noncoding strand, and cleavage was carried out under conditions that favored single-stranded (pH 5.5, lanes 1 and 3) or double-stranded (pH 7.5, lanes 2 and 4) DNA cleavage. Reactions were performed in the presence of calcium (lanes 1 and 2) or magnesium (lanes 3 and 4) as described under Experimental Procedures. An autoradiogram is shown.

ble-stranded breaks in DNA, it covalently attaches to both 5′-termini of the cleaved nucleic acid (Sander & Hsieh, 1983; Liu et al., 1983). Considering that the enzyme is capable of creating single-stranded breaks in duplex DNA (Figure 1) and that the active form of the enzyme is a homodimer (Sander & Hsieh, 1983; Shelton et al., 1983; Miller et al., 1981; Goto et al., 1984; Halligan et al., 1985; Schomburg & Grosse, 1986), it is likely that each subunit of topoisomerase II is responsible for breaking/religating one of the two strands of its DNA substrate, rather than one enzyme subunit acting on both strands. To test this, products of a double-stranded DNA cleavage reaction (i.e., topoisomerase II–linear DNA complexes) were examined by electron microscopy. The denaturing conditions employed (0.025% SDS, 2 min at 75 °C) dissociate topoisomerase II subunits but do not break covalent bonds.

Typical micrographs are shown in Figure 6. As can be seen, both ends of linear DNA molecules were associated with separate enzyme subunits. To ensure that the results of Figure 6 were statistically relevant, 13 different fields of view were scored. Of the 108 linear DNA molecules found, 98 were complexed with protein on both ends. None of the remaining 10 molecules was associated with protein on either end. A comparable number of protein-free linear molecules was observed in 13 control fields of DNA in assay buffer that contained no topoisomerase II. These findings indicate that each strand of the DNA double helix is nicked by a separate polypeptide of the enzyme.

Kinetic Pathway of DNA Religation by Topoisomerase II. All of the above results are consistent with the suggestion that topoisomerase II mediates double-stranded cleavage by making two coordinated single-stranded cuts in the nucleic acid backbone (Osheroff & Zechiedrich, 1987). To this end, an attempt was made to characterize the kinetic pathway of the forward DNA cleavage reaction of *Drosophila* topoisomerase II. Unfortunately, this experiment was not feasible because the reaction reached equilibrium within 5 s, the shortest time point that could be accurately performed using conventional methodologies. Therefore, the mechanism of enzyme-mediated DNA religation was examined. This was possible since the time course (Figure 7) of religation (under the assay conditions employed) was slow enough to be accurately and reproducibly monitored.

The DNA religation assay takes advantage of the fact that calcium can be used to trap a kinetically competent covalent topoisomerase II–DNA cleavage complex (Osheroff & Zechiedrich, 1987). Treatment of this complex with proteinase K frees linear DNA (in which both strands were cleaved) and nicked molecules (in which only one strand was cleaved) in an ~50:50 ratio (Figure 7, second lane). The assay was carried out by a four-step procedure. (1) To establish a DNA cleavage equilibrium, topoisomerase II was incubated with negatively supercoiled pBR322 plasmid DNA and divalent calcium ions in 100 mM salt, buffered to pH 7.9 with Tris-HCl, at 30 °C. (2) EDTA was added to chelate the divalent cation and trap the active topoisomerase II–DNA cleavage complex. (3) The salt concentration in the assay was increased from 100 to 200 mM to prevent recleavage of the DNA during the course of religation (Liu et al., 1983; Osheroff & Zechiedrich, 1987). (4) Religation was initiated (on ice to slow reaction rates) by the addition of excess magnesium ions. The final product of this assay was the original supercoiled pBR322 DNA substrate (last lane). Results similar to those described

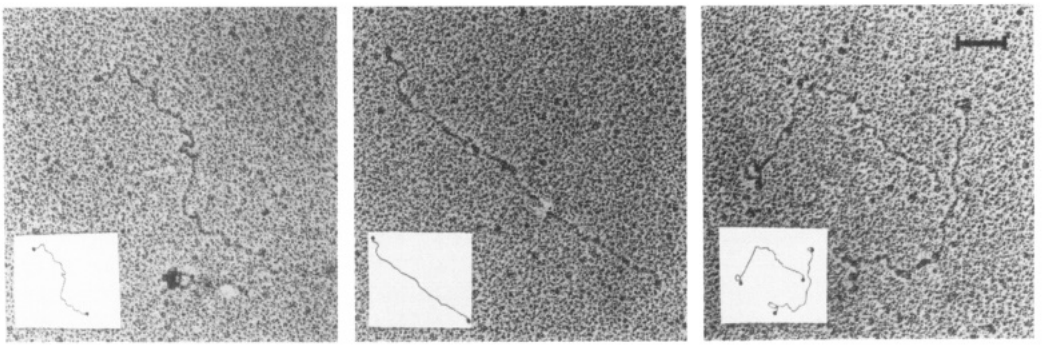

FIGURE 6: Electron microscopy of topoisomerase II–linear DNA cleavage products. Assays contained 25 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA and were carried out at pH 7.5 in the presence of calcium. Cleavage was terminated by the addition of 5 mM EDTA (final concentration), followed by 0.025% SDS (final concentration) (45). Reaction products were diluted ~15-fold and prepared for electron microscopy as described under Experimental Procedures. Bar = 200 Å.

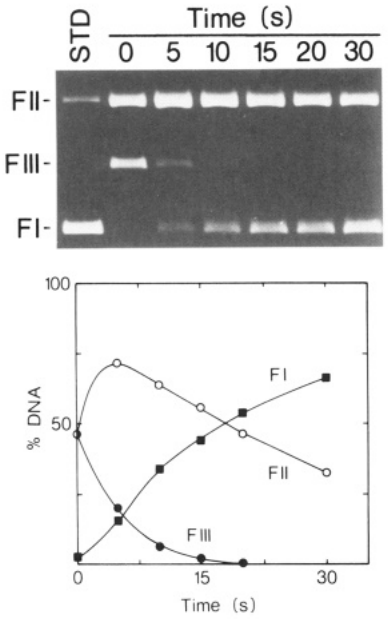

FIGURE 7: Time course of enzyme-mediated religation of cleaved DNA. Assays were carried out in Tris-HCl, pH 7.9, as described under Experimental Procedures. Covalent topoisomerase II–DNA cleavage products generated in calcium-containing reactions were trapped by the addition of EDTA. Religation was initiated by the addition of magnesium at time zero. The top panel shows reaction products subjected to electrophoresis on a 1% agarose gel at the indicated times following the addition of magnesium. Results are depicted graphically in the bottom panel. Linear DNA (FIII, ●), nicked DNA (FII, ○), and supercoiled DNA (FI, ■) are plotted as the percentage of the total DNA present in the reaction mixture.

below were also found when reactions were carried out in either Tris-HCl or imidazole–HCl, pH 7.5.

A time course for DNA religation is shown in the upper panel of Figure 7 and depicted graphically in the lower panel. During the course of the reaction, levels of linear DNA (FIII) dropped rapidly. The drop in linear DNA was accompanied by a transient rise in nicked circular molecules (FII). Following an initial lag, the rate of regeneration of supercoiled plasmid molecules (FI) was proportional to the rate of disappearance of nicked DNA. The reaction profile described above is diagnostic of a two-step sequential mechanism (Segel, 1975; Fersht, 1985) in which nicked circular DNA is an obligatory kinetic intermediate in the topoisomerase II mediated religation of double-stranded DNA breaks (i.e., FIII → FII → FI).

Apparent First-Order Rate Constants for the Topoisomerase II Mediated DNA Religation Reaction. The first-order rate constants for the enzymatic religation of cleaved DNA were calculated from a semilogarithmic plot of percent DNA versus time (Segel, 1975; Fersht, 1985) (Figure 8). The disappearance of doubly cleaved (FIII) and nicked (FII) DNA was linear with respect to time. As determined from the slopes of the respective plots, the apparent first-order rate constant for the conversion of linear to nicked DNA was 0.2 s^{-1} and for nicked to supercoiled was 0.03 s^{-1}. Thus, under the reaction conditions employed, the rate of religation of the first strand was ~6-fold faster than the rate of religation of the second strand. Finally, the apparent rate constant for the regeneration of negatively supercoiled DNA (FI) was 0.03 s^{-1}, as calculated from a semilogarithmic plot of the difference between the percent FI DNA at the completion of the reaction (at t_∞, FI ≈ 100%) and the percent FI DNA at any reaction time (t) versus time (Figure 8). Since this value is identical with that for the disappearance of FII, the conversion of nicked to supercoiled DNA (i.e., the resealing of the second strand) must be the rate-limiting step of the religation reaction.

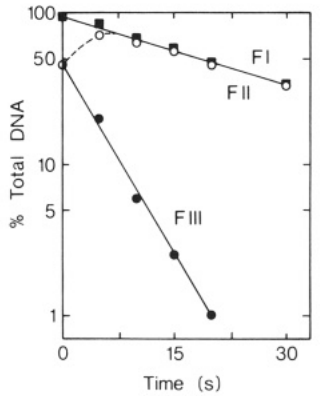

FIGURE 8: Semilogarithmic plot of percent DNA versus time for the topoisomerase II mediated religation reaction. Conditions are as described in the caption of Figure 7. The regeneration of negatively supercoiled DNA was plotted as the difference between the percent FI DNA at the completion of the reaction ($t = \infty$) and the percent DNA at any reaction time (t). Linear DNA (FIII, ●), nicked DNA (FII, ○), and supercoiled DNA (FI, ■) are shown.

Discussion

A simplified kinetic scheme for the double-stranded DNA cleavage/religation reaction of *D. melanogaster* topoisomerase II is proposed below:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E{\cdot}S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–}S_I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}S_{II}$$

E represents the enzyme, S represents the double-stranded DNA substrate, S_I represents DNA containing a single-stranded break (i.e., nick), S_{II} represents DNA with a double-stranded break, the dot denotes a noncovalent interaction, and the dash represents a covalent complex. This scheme encompasses the previous findings that topoisomerase II is covalently joined to cleaved DNA molecules, that cleavage/religation is an equilibrium process which can be reversed by the addition of salt, and that ATP is not required for the reaction (Sander & Hsieh, 1983; Liu et al., 1983; Osheroff & Zechiedrich, 1987). On the basis of the data presented above, the reaction mechanism of double-stranded DNA cleavage/religation has been extended to include an obligatory topoisomerase II–nicked DNA intermediate $E\text{–}S_I$.

Several lines of evidence support the above scheme. First, reaction conditions that produce double-stranded DNA cleavage invariably generate nicked DNA products as well (Nelson et al., 1984; Tewey et al., 1984; Pommier et al., 1985; Liu et al., 1983; Osheroff & Zechiedrich, 1987; Muller et al., 1988).

Second, under conditions (i.e., pH 5.0) that generated high levels of enzyme-mediated single-stranded DNA cleavage, the nicking reaction exhibited covalent topoisomerase II–DNA linkage and salt reversibility. Moreover, the sequence specificity of DNA nicking was identical with that of double-stranded cleavage. The fact that the $E\text{–}S_I$ complex was produced with all the hallmarks of the double-stranded DNA cleavage complex ($E\text{–}S_{II}$) effectively precludes a reaction mechanism in which the enzyme makes only concerted double-stranded breaks in the nucleic acid backbone.

Third, electron microscopy studies revealed that both ends of doubly cleaved (i.e., linear) DNA molecules were complexed with separate polypeptides. Therefore, each single-stranded DNA break appears to be mediated by a separate subunit of the homodimeric (Sander & Hsieh, 1983; Shelton et al., 1983) *Drosophila* enzyme. A similar finding has been reported for

the multimeric type II topoisomerase from bacteriophage T4 (Kreuzer & Huang, 1983).

Finally, nicked DNA was found to be an obligatory kinetic intermediate in the religation of double-stranded DNA breaks. Under the conditions employed, the apparent first-order rate constant for the religation of the first strand (0.2 s^{-1}) was ~6-fold faster than the constant for the religation of the second strand (0.03 s^{-1}). Thus, at least during religation, the enzyme acts in a sequential manner and the reunion of the second break is the rate-limiting step of the reaction.

A reaction scheme similar to that shown above has been postulated for several type II restriction endonucleases. *Eco*R1, *Hpa*II, *Hin*dIII, and *Hha*II all have been shown to proceed through an obligatory single-strand intermediate in their kinetic pathways to doubly cleaved DNA (Ruben et al., 1977; Rubin & Modrich, 1978; Halford et al., 1979; Halford & Johnson, 1983; Kaddurah-Daouk et al., 1985). Although *Bam*HI and *Eco*RV produce no nicked DNA molecules under optimal reaction conditions, singly cut intermediates were observed under suboptimal reaction conditions (Halford et al., 1979; Potter & Eckstein, 1984; Halford & Goodall, 1988). Presumably, these latter enzymes act by making two sequential single-stranded breaks in their nucleic acid substrates, but under optimal conditions either $k_2 \gg k_1$ or the rate-limiting step precedes DNA cleavage. Thus far, the only type II restriction endonuclease for which this kinetic scheme cannot be applied is *Sal*I. Under no circumstances has an obligatory nicked DNA intermediate been demonstrated (Potter & Eckstein, 1984; Maxwell & Halford, 1982). Therefore, *Sal*I appears to cleave DNA by making simultaneous rather than sequential single-stranded breaks.

At acidic pH (≤6.0), several restriction endonucleases produce increased levels of singly cleaved DNA (Halford & Goodall, 1988; Maxwell & Halford, 1982). It has been proposed for *Eco*RV that this nicking results from a decreased affinity of the system for magnesium ions (Halford & Goodall, 1988). Hence, at any given time, only one subunit of the homodimeric enzyme is magnesium bound and active in DNA cleavage. This does not appear to be the case for topoisomerase II, since the enzyme's affinity for magnesium at pH 5.0 is at least as great as that observed at pH 7.5. In addition, topoisomerase II mediated DNA nicking is not caused by dissociation of enzyme subunits. Finally, neither K_s nor the k_1/k_{-1} ratio can be greatly affected at low pH since total levels of cleavage at pH 5.0 were similar to those observed at pH 7.5. Thus, acidic pH probably shifts the DNA cleavage/religation equilibrium from double-stranded cleavage to single-stranded nicking by decreasing the k_2/k_{-2} ratio. This conclusion is supported by experiments that examined the kinetic profile of enzyme-mediated DNA religation at pH 5.0 (not shown). In these experiments, the rate of disappearance of linear DNA molecules (which presumably relfects k_{-2}) was ~13-fold faster than the rate of disappearance of nicked DNA (which presumably reflects k_{-1}). This is as compared to the ~6-fold difference observed at pH 7.5.

The DNA cleavage reaction of eukaryotic topoisomerase II has been the subject of intense interest. This is due largely to the fact that the antineoplastic activities of several clinically relevant chemotherapeutic drugs correlate with their abilities to stabilize topoisomerase II–DNA cleavage complexes (Zwelling, 1985; Glisson & Ross, 1987). Clearly, before the mechanisms of these drugs can be delineated, the details of their target reaction must be well-defined. While other aspects of the reaction still need to be addressed, the results of this study indicate that topoisomerase II mediates double-stranded DNA cleavage/religation by making two sequential single-stranded breaks in the backbone of its nucleic acid substrate.

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Registry No. DNA topoisomerase, 80449-01-0.

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