Strand Specificity of the Topoisomerase II Mediated Double-Stranded DNA Cleavage Reaction†

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ABSTRACT: The strand specificity of topoisomerase II mediated DNA cleavage was analyzed at the nucleotide level by characterizing the enzyme's interaction with a strong DNA recognition site. This site was isolated from the promoter region of the extrachromosomal rRNA genes of *Tetrahymena thermophila* and was recognized by type II topoisomerases from a variety of phylogenetically diverse eukaryotic organisms, including *Drosophila*, *Tetrahymena*, and calf thymus. When incubated with this site, topoisomerase II was found to introduce single-stranded breaks (i.e., nicks) in addition to double-stranded breaks in the nucleic acid backbone. Although the nucleotide position of cleavage on both the noncoding and coding strands of the rDNA remained unchanged, the relative ratios of single- and double-stranded DNA breaks could be varied by altering reaction conditions. Under all conditions which promoted topoisomerase II mediated DNA nicking, the enzyme displayed a 3–10-fold specificity for cleavage at the noncoding strand of its recognition site. To determine whether this specificity of topoisomerase II was due to a faster forward rate of cleavage of the noncoding strand or a slower rate of its religation, a DNA religation assay was performed. Results indicated that both the noncoding and coding strands were religated by the enzyme at approximately the same rate. Therefore, the DNA strand preference of topoisomerase II appears to be embodied in the enzyme's forward cleavage reaction.

Type II topoisomerases have been purified from a wide range of eukaryotic sources spanning protozoa, fungi, plants, insects, vertebrates, and mammals [for a review, see Wang (1985)]. These ubiquitous enzymes are involved in many aspects of nucleic acid structure and metabolism and are able to catalyze the interconversion of several different topological states of DNA (Wang, 1985). Among the in vitro reactions which have been ascribed to eukaryotic topoisomerase II are the relaxation of supercoiled DNA and the knotting/unknotting and catenation/decatenation of circular duplex molecules [for reviews, see Vosberg (1985), Maxwell and Gellert (1986), and Osheroff (1989)]. Central to all these reactions is the enzyme's ability to introduce a transient double-stranded break in the backbone of its nucleic acid substrate.

Because of its importance for enzyme function, the DNA cleavage/religation reaction of topoisomerase II has been the subject of many studies (Wang, 1985; Osheroff, 1989). During cleavage, topoisomerase II forms a covalent complex with its DNA substrate in which the enzyme is attached via an O^4-phosphotyrosine bond to the 5′ termini of the cleaved DNA (Sander & Hsieh, 1983; Liu et al., 1983; Rowe et al., 1986; Osheroff & Zechiedrich, 1987). In addition, cleavage on the opposite strands of duplex DNA is always offset by four bases, generating molecules with 5′-protruding ends (Sander & Hsieh, 1983; Liu et al., 1983). A mechanistic study (Zechiedrich et al., 1989) indicates that the enzyme cleaves double-stranded DNA by creating two sequential single-stranded breaks (i.e., nicks), rather than one concerted double-stranded break, in the nucleic acid backbone. Moreover, during this reaction, each strand of the DNA is cleaved by a separate subunit of topoisomerase II. Finally, under appropriate conditions, cleavage of the first strand can be uncoupled from that of the second, such that the enzyme introduces predominantly nicks, rather than double-stranded breaks, in the DNA. Topoisomerase II mediated DNA nicking has also recently been observed with the enzyme purified from chicken blood cells (Muller et al., 1988).

Topoisomerase II acts at preferred sites in DNA (Wang, 1985). On the basis of kinetic and binding data, sites of cleavage defined in vitro appear to be sites of catalytic action (Udvardy et al., 1986; Sander et al., 1987). The interaction of topoisomerase II with DNA has been examined at the nucleotide level, and a weak consensus recognition sequence has been reported for both the Drosophila (Sander & Hsieh, 1985) and the chicken (Spitzner & Muller, 1988) enzyme. Despite the homodimeric structure of eukaryotic topoisomerase II (Wang, 1985), all DNA recognition sites examined to date appear to be asymmetric (i.e., the two strands of the cleavage site have different nucleotide sequences). This is in marked contrast to the palindromic cleavage sites of most homodimeric type II restriction endonucleases (Kessler & Holtke, 1986).

Together with the finding that topoisomerase II cleaves double-stranded DNA by making two coordinated single-stranded breaks, the asymmetric nature of the enzyme's recognition site suggests that within this site, topoisomerase II may be able to discriminate between the two complementary strands of the DNA helix. Therefore, the nucleotide strand specificity of the eukaryotic type II enzyme was investigated. This was accomplished by isolating and characterizing a strong topoisomerase II DNA cleavage site from the promoter region of the extrachromosomal rRNA genes of *Tetrahymena*

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thermophila. Results of this study indicate that at cleavage/religation equilibrium, the enzyme displays an intrinsic preference (3–10-fold) for the noncoding over the coding strand of the rDNA site. This specificity is embodied in the enzyme's forward cleavage, rather than religation reaction. The ability of topoisomerase II to discriminate between the two strands of its DNA recognition site provides additional insight into the mechanism of the enzyme's catalytic function.

Experimental Procedures

Purification of Topoisomerase II. Insect DNA topoisomerase II was purified from the nuclei of *Drosophila* Kc tissue culture cells by the procedure of Shelton et al. (1983).

Mammalian topoisomerase II was purified from calf thymus glands according to the procedure of Schomburg and Grosse (1986) through the polymin P precipitation step. Further purification was according to Shelton et al. (1983).

Protozoan topoisomerase II was purified from cultures ($\sim 10^5$ cells/mL) of *T. thermophila* strain B1868-7. Macronuclei were isolated as described by Gocke et al. (1978) except that 0.2 mM phenylmethanesulfonyl fluoride was added to the solutions. Nuclear proteins were extracted by the procedure of Thomsen et al. (1987). The extract was centrifuged for 10 min at 12000*g*, and the resulting supernatant was chromatographed on a Bio-Rex 70 column according to Halligan et al. (1985). The column was step-eluted with a buffer containing 0.7 M potassium phosphate, pH 7.1, 10% glycerol, 0.5 mM ethylenediaminetetraacetic acid (EDTA),[1] 1 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride. The final Bio-Rex 70 pool was diluted 2.5 times and applied to a hydroxylapatite column equilibrated with 0.2 M potassium phosphate. The column was developed with a linear gradient of 0.2–0.7 M potassium phosphate, pH 7.1. The peak of topoisomerase II activity eluted between 0.4 and 0.45 M potassium phosphate. The hydroxylapatite pool was loaded onto a phenyl-Sepharose column equilibrated in 0.4 M potassium phosphate, pH 7.1, and eluted with 50% ethylene glycol, 20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, and 1 mM dithiothreitol.

Plasmids. Plasmid pUTtrII contained a *Dde*I fragment of *T. thermophila* rDNA spanning from nucleotide position −967 to −635 with respect to the initiation point of transcription (Karrer & Gall, 1976). The plasmid was constructed by filling in the recessed *Dde*I 3′ termini with Klenow enzyme and inserting the fragment into the *Hinc*II site located in the polylinker region of pUC19 (Yanish-Perron et al., 1985).

Gel Electrophoresis. The products of topoisomerase II mediated cleavage of negatively supercoiled pBR322 DNA were assessed by electrophoresis on a 1% agarose gel. Samples were subjected to electrophoresis in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA at 5 V/cm. Gels were stained for 30 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 using Polaroid type 665 positive/negative film.

DNA sequencing reactions and the products of topoisomerase II mediated cleavage of linear rDNA fragments were analyzed on denaturing polyacrylamide gels [6% (w/v) acrylamide/0.3% (w/v) *N,N*-methylenebis(acrylamide), and 8 M urea] run for 2–4 h (40 V/cm) in 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.3 (TBE). Alternatively, topoisomerase II DNA cleavage products were analyzed on native polyacrylamide gels [10% (w/v) acrylamide/0.35% (w/v) *N,N*-methylenebis(acrylamide)] run for 10 h (6 V/cm) in TBE or [4% (w/v) acrylamide/0.14% (w/v) *N,N*-methylenebis(acrylamide)] run for 4 h (15 v/cm) in TBE. Reaction products were visualized by autoradiography using Kodak XAR film with or without a Cronex Lightning Plus Screen (Du Pont). In some cases, DNA fragments were excised from native polyacrylamide gels and were reanalyzed on denaturing polyacrylamide gels. To this end, radioactive bands of interest were localized by autoradiography and excised. Following excision, DNA was extracted by grinding the gel slices followed by heating at 60 °C for 12 h in 100 μL of 1 mM Tris-HCl, pH 8, and 0.1 mM EDTA. Gel pieces were removed by centrifugation, and DNA samples were concentrated to a volume of ~5 μL under reduced pressure. Samples were applied to the denaturing polyacrylamide gel system described above.

Isolation of DNA Restriction Fragments, End-Labeling, and Sequence Analysis. Plasmid pUTtrII was digested with *Bam*HI and *Hin*dIII (New England Biolabs, Boehringer), and protruding 5′ ends were dephosphorylated by treatment with alkaline phosphatase (Boehringer). The resulting two DNA fragments were separated by agarose gel electrophoresis, localized by ethidium bromide staining, and the band resulting from the 365 bp *Bam*HI–*Hin*dIII fragment (containing the rDNA topoisomerase II recognition site) was excised from the gel. The DNA was purified from the gel slice by using a "Gene Clean" kit (Bio 101). The DNA fragment was labeled on both protruding 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). Single-end-labeled fragments for sequencing or cleavage reactions were obtained from the double-end-labeled fragment by digestion with appropriate restriction enzymes which cut close to one or the other end of the fragment. Sequencing reactions were performed according to the procedure of Maxam and Gilbert (1980) (the G and C > T reactions) and by a modification of this method (Bencini et al., 1984) (A + G and A + C reactions).

Topoisomerase II Mediated DNA Cleavage Reactions. End-labeled DNA restriction fragments which contained the rDNA cleavage site (0.15 pmol) were incubated with topoisomerase II (3 pmol) in 50 μL of 10 mM buffer (Tris-HCl, pH 7.5, or imidazole, pH 5.5), 5 mM divalent cations ($MgCl_2$ or $CaCl_2$), 25 mM NaCl, and 0.1 mM EDTA (cleavage buffer) at 30 °C for 6 min. The cleavage reaction was terminated by the addition of SDS followed by EDTA to final concentrations of 1% and 10 mM, respectively. In control reactions, NaCl (0.4 M final concentration) was added prior to SDS and EDTA. Following proteinase K digestion (Merck; 300 μg/mL, 60 min at 37 °C), 1 volume of deionized formamide, 0.05% bromphenol blue, 0.03% xylene cyanole, and 5 mM EDTA, pH 8.5, were added, and samples were applied to denaturing or native polyacrylamide gels. When DNA was loaded on native polyacrylamide gels, the formamide was replaced with 30% glycerol.

When negatively supercoiled pBR322 plasmid DNA was used as a substrate for topoisomerase II mediated cleavage, reactions contained 2 pmol of enzyme and 0.1 pmol of plasmid DNA in 20 μL of cleavage buffer. Samples were otherwise treated as described above, except that the reaction products were mixed with loading buffer (2.5 μL of 60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole, and 10 mM Tris-HCl, pH 7.9) and resolved by agarose gel electrophoresis.

Topoisomerase II Mediated DNA Religation Reaction. The religation assay of Osheroff and Zechiedrich (1987) was

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; bp, base pair(s).

employed. Topoisomerase II (3 pmol) was incubated with the double-end-labeled *Bam*HI–*Hin*dIII fragment (0.15 pmol) in 50 μL of cleavage buffer (pH 7.5), which contained 5 mM $CaCl_2$. Kinetically competent covalent topoisomerase II–DNA complexes were trapped by the addition of 2.5 μL of 0.2 M EDTA. NaCl (0.4 M final concentration) was added to prevent recleavage of the DNA during the religation reaction (Osheroff & Zechiedrich, 1987), and samples were placed on ice to slow religation rates. Religation was initiated by the addition of cold $MgCl_2$ (7.5 mM final concentration). SDS (5 μL of a 10% solution) was added to terminate religation at intervals of 2–5 s. Samples were digested with proteinase K (300 μg/mL, 12 h), phenol extracted, and ethanol precipitated. The DNA was resuspended and subjected to electrophoresis on either denaturing or native polyacrylamide gels as described above.

Quantitation of DNA Cleavage Products on Polyacrylamide Gels. The relative frequency of cleavage was determined by densitometric scanning of the autoradiograms using a Shimadzu dual-wavelength thin-layer chromatoscanner, Model CS930.

Results

Isolation of a Strong Topoisomerase II DNA Cleavage Site. In order to characterize the interaction of eukaryotic topoisomerase II with DNA at the nucleotide level, a strong site for enzyme-mediated cleavage was isolated from the promoter region of the extrachromosomal rRNA genes of *T. thermophila*. This region, which also contains an origin for DNA replication (Cech & Brehm, 1981), was examined for topoisomerase II sites because of its relative abundance of specific recognition sequences for type I topoisomerases (Bonven et al., 1985). On the basis of in vitro cleavage assays, at least two strong topoisomerase II recognition sites were found (not shown). A DNA fragment containing one of these sites was cloned into plasmid pUC19, creating plasmid pUTtrII. A detailed cloning strategy is given under Experimental Procedures.

A 365 bp linear *Bam*HI–*Hin*dIII fragment spanning the rDNA insert was isolated from plasmid pUTtrII, radioactively labeled at both protruding 5′ termini, and used as a DNA cleavage substrate for *Drosophila*, calf thymus, and *Tetrahymena* topoisomerase II. Sodium dodecyl sulfate (SDS) was added to trap covalent enzyme–DNA cleavage products (Vosberg, 1985). Topoisomerase II was digested with proteinase K, and samples were analyzed on a native polyacrylamide gel (Figure 1). Only one topoisomerase II mediated double-stranded DNA cleavage site was observed for all three enzymes, as evidenced by the conversion of the 365 bp DNA substrate to two fragments of 222 and 143 bp, respectively.

Nucleotide Sequence of a Strong Topoisomerase II DNA Cleavage Site. To delineate the nucleotide sequence of the topoisomerase II DNA cleavage site, two single-end-labeled fragments were generated from the double-end-labeled *Bam*HI–*Hin*dIII fragment. A 276 bp fragment specifically end-labeled on the noncoding strand was produced by digestion with restriction endonuclease *Xba*I (Figure 2, lane 2 and bottom). Digestion with *Dra*I generated a 271 bp fragment specifically end-labeled on the coding strand (Figure 2, lane 14 and bottom).

Both single-end-labeled fragments were used as substrates in topoisomerase II mediated cleavage assays. Reaction products were analyzed on a denaturing polyacrylamide gel (Figure 2) in parallel with the Maxam–Gilbert degradation reactions of the corresponding fragment (noncoding, lanes 3–6; coding, lanes 10–13). Only one major site of cleavage was observed on either strand, as evidenced by the emergence of single bands in lanes 7 (noncoding) and 9 (coding). The same sites of cleavage were obtained with the original double-end-labeled 365 bp fragment (lane 8).

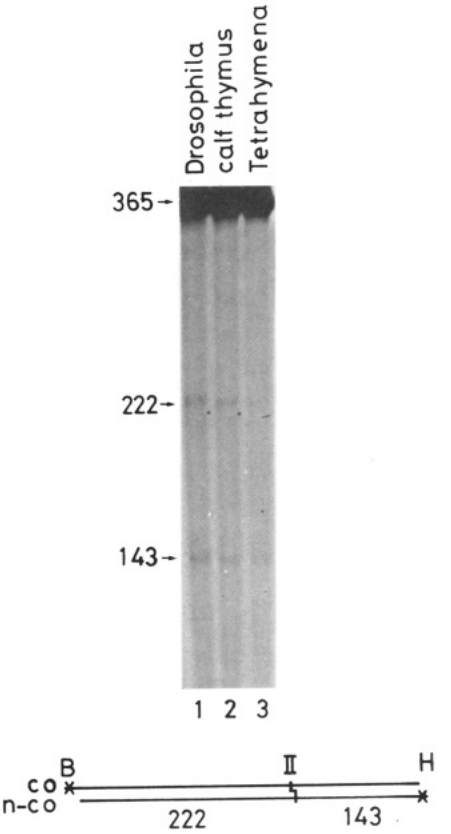

FIGURE 1: Cleavage of an rDNA fragment by type II topoisomerases from phylogenetically distant organisms. Topoisomerase II cleavage assays were carried out in the presence of 5 mM $MgCl_2$ at pH 7.5 as described under Experimental Procedures, and reaction products were analyzed on a 4% native polyacrylamide gel. The bottom diagram shows the DNA fragment used in the cleavage assays: II, topoisomerase II cleavage site; B, *Bam*HI; H, *Hin*dIII; co, coding strand; n-co, noncoding strand; asterisk, radioactive label. The numbers at the left give the sizes of DNA fragments in base pairs.

The complete structure of the topoisomerase II double-stranded rDNA recognition site is depicted in Figure 3. Enzyme-mediated cleavage takes place between the indicated G and C residues on the noncoding strand and the A and T residues on the coding strand. As generally shown for topoisomerase II (Sander & Hsieh, 1983, 1985; Liu et al., 1983), cleavage at this site generates a DNA product with a four-base 5′ overhang. The rDNA cleavage site has an asymmetric structure. However, a palindromic 6 bp recognition sequence for the restriction endonuclease *Sph*I is located immediately upstream from the axis of topoisomerase II mediated cleavage. The site of *Sph*I cleavage coincides with that of topoisomerase II on the noncoding strand, but is offset by eight bases on the coding strand.

Strand Specificity of Topoisomerase II Mediated DNA Cleavage. Experiments designed to characterize the DNA strand specificity of topoisomerase II took advantage of the fact that the enzyme creates single-stranded breaks (i.e., nicks) in duplex DNA as an intermediate in its double-stranded DNA cleavage reaction (Zechiedrich et al., 1989). By comparing the relative degree of nicking on the two complementary strands of a specific topoisomerase II cleavage site, it was possible to determine if the enzyme displayed a preference for one strand over the other.

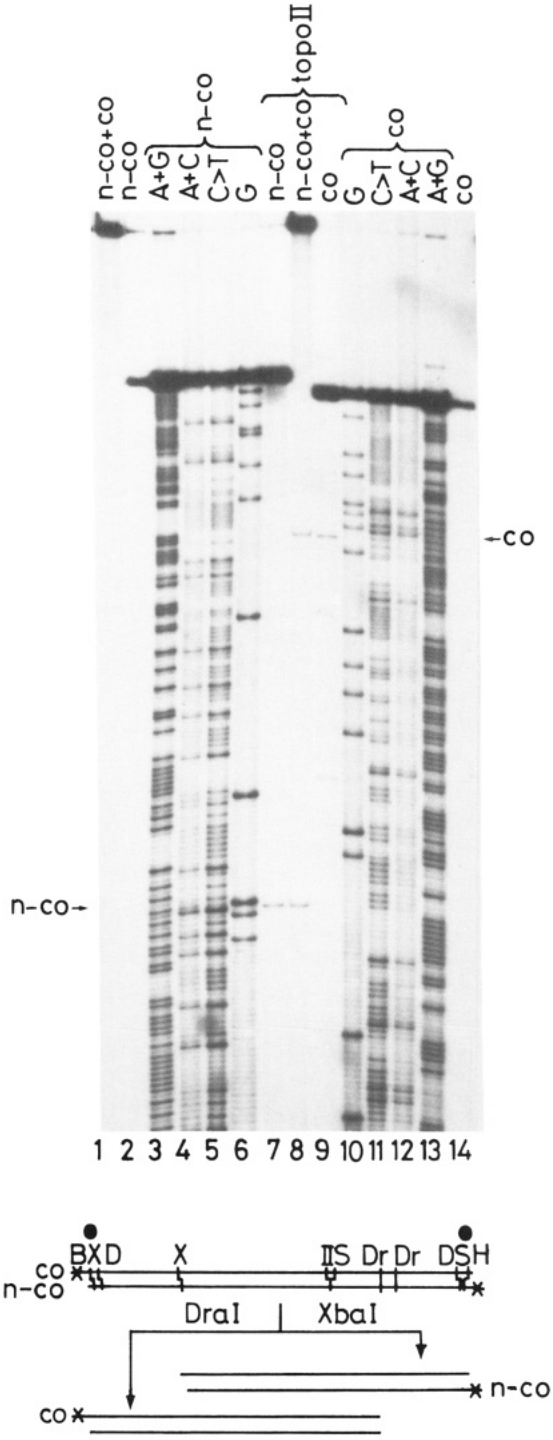

FIGURE 2: Nucleotide sequencing of the rDNA cleavage site for topoisomerase II. Topoisomerase II cleavage assays were carried out in the presence of 5 mM $MgCl_2$ at pH 7.5 as described under Experimental Procedures. Reaction products were analyzed on a 6% denaturing polyacrylamide gel. The DNA fragments incubated with topoisomerase II are the double-end-labeled *Bam*HI–*Hin*dIII fragment (lane 8), the double-end-labeled fragment digested with *Xba*I (lane 7), and the double-end-labeled fragment digested with *Dra*I (lane 9). The corresponding untreated fragments are shown in lanes 1, 2, and 14, respectively. The *Xba*I–*Hin*dIII fragment (labeled at the noncoding strand) and the *Bam*HI–*Dra*I fragment (labeled at the coding strand) were subjected to Maxam–Gilbert sequencing reactions in lanes 3–6 and 10–13, respectively. Lanes 3 and 13, A + G; lanes 4 and 12, A + C; lanes 5 and 11, C > T; lanes 6 and 10, G. Arrows indicate the positions of cleavage on the coding and noncoding strands. The bottom diagram shows the DNA fragments used in the cleavage assays: II, topoisomerase II cleavage site; B, *Bam*HI; H, *Hin*dIII; X, *Xba*I; D, *Dde*I; Dr, *Dra*I; S, *Sph*I; co, coding strand; n-co, noncoding strand; asterisk, radioactive label. Ẋ and Ṡ are cleaved only to a low extent, due to their locations close to the ends of the fragment.

FIGURE 3: Structure of a eukaryotic topoisomerase II DNA cleavage site located in the promoter region of the *Tetrahymena* rRNA gene. The four-base stagger resulting from topoisomerase II mediated cleavage on the noncoding (n-co) and coding (co) strands is illustrated by a solid line.

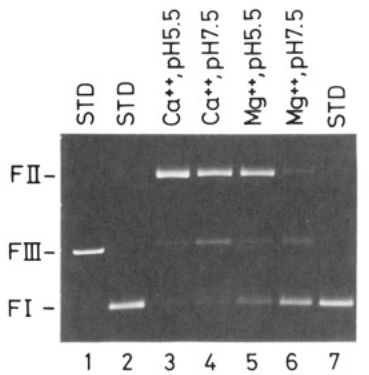

FIGURE 4: Double- and single-stranded cleavage of negatively supercoiled pBR322 DNA by Drosophila topoisomerase II. Topoisomerase II cleavage assays were carried out as described under Experimental Procedures, and reaction products were analyzed on a 1% agarose gel (lanes 3–6). Lane 1, pBR322 DNA linearized by cleavage with *Eco*RI; lane 2, negatively supercoiled DNA standard incubated in 5 mM $CaCl_2$, pH 5.5, without enzyme; lane 7, negatively supercoiled DNA standard incubated in 5 mM $MgCl_2$, pH 7.5, without enzyme. The positions of form I (FI), fully supercoiled DNA; form II (FII), nicked circular plasmid molecules; and form III (FIII), linear molecules, are shown.

The level of nicked products generated by topoisomerase II cleavage assays was altered by varying the divalent cation used to promote the enzyme-mediated reaction and the pH of reaction mixtures (Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989). The effect of assay conditions on the DNA cleavage reaction of *Drosophila* topoisomerase II is demonstrated in Figure 4. This experiment utilized a negatively supercoiled plasmid substrate (lane 2), which allowed the simultaneous visualization of double-stranded cleavage [i.e., conversion of negatively supercoiled (FI) to linear (FIII) molecules] and nicking [i.e., conversion of FI to nicked (FII) molecules]. At pH 7.5 in magnesium-containing buffers, topoisomerase II created predominantly double-stranded breaks in DNA (lane 6). When the pH of the reaction was lowered to 5.5, a considerably higher proportion of DNA nicking was observed (lane 5). Similar results were obtained when magnesium was replaced by calcium, except that levels of DNA nicking were consistently higher (compare lanes 3 and 4 to lanes 5 and 6).

The reaction conditions described above were used in conjunction with the specific rDNA cleavage site in order to examine the strand specificity of *Drosophila* topoisomerase II. Studies employed the double-end-labeled *Bam*HI–*Hin*dIII fragment, so that the relative cleavage of both the noncoding and coding strands could be monitored in a single experiment (see Figure 2, lane 8). The incorporation of radioactive label at both ends of the fragment was comparable, as determined by densitometric scanning of a *Dra*I digest of the original fragment (Figure 5B, lane 6; see the bottom of Figure 2 for a restriction map of the fragment). Thus, relative differences in the intensities of the two cleavage bands accurately reflect differences in the levels of topoisomerase II mediated cleavage at the noncoding or coding strand, rather than a disproportionate distribution of label.

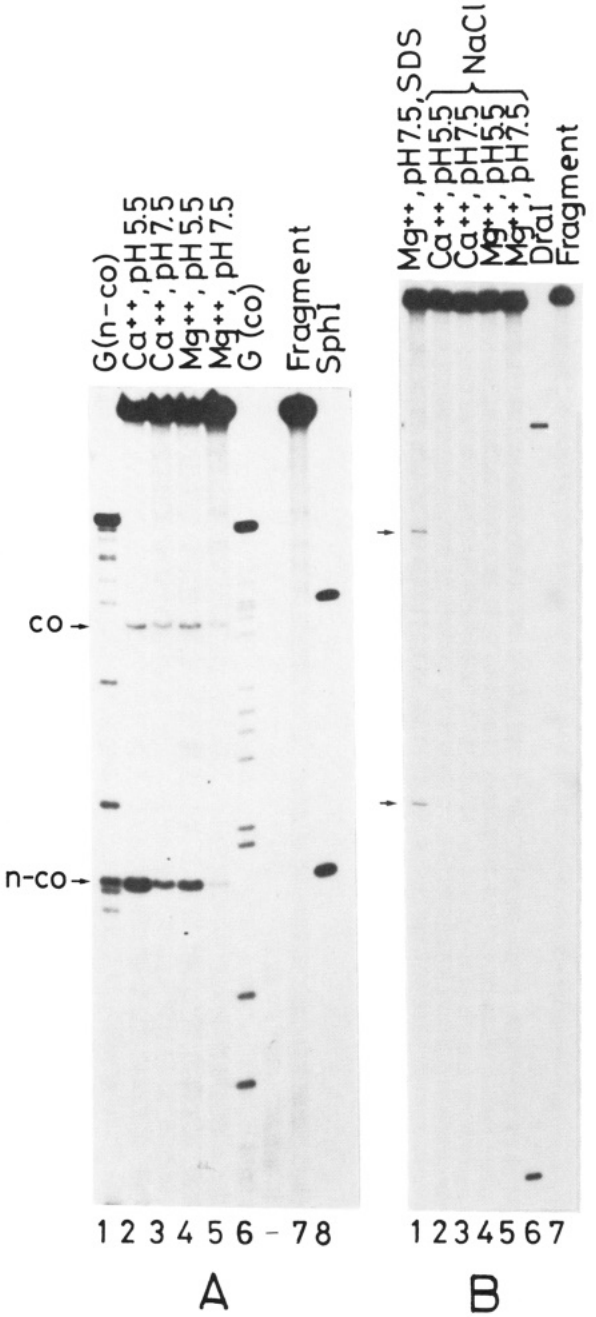

FIGURE 5: Double- and single-stranded cleavage of the rDNA site by *Drosophila* topoisomerase II. (A) Topoisomerase II mediated cleavage of the double-end-labeled *Bam*HI–*Hin*dIII fragment was carried out as described under Experimental Procedures. Reaction products were analyzed on a 6% denaturing polyacrylamide gel (lanes 2–5). As markers are shown Maxam–Gilbert G reaction of the 5′-single-end-labeled *Xba*I–*Hin*dIII fragment (n-co; lane 1), Maxam–Gilbert G reaction of the 5′-single-end-labeled *Bam*HI–*Dra*I fragment (co; lane 6), the untreated double-end-labeled *Bam*HI–*Hin*dIII fragment (lane 7), and an *Sph*I digest of the double-end-labeled fragment (lane 8). The cleavage positions are indicated by arrows. (B) Salt reversibility experiments were carried out as described under Experimental Procedures. Reaction products were analyzed on a 6% denaturing polyacrylamide gel (lanes 2–5). Lane 1 shows the cleavage positions on the double-end-labeled *Bam*HI–*Hin*dIII fragment, lane 6 shows a *Dra*I digestion of the fragment, and lane 7 shows the fragment incubated in the presence of 5 mM $CaCl_2$, pH 5.5, without enzyme.

As analyzed by electrophoresis on a denaturing polyacrylamide gel (Figure 5A), the nucleotide position of enzyme-mediated cleavage on the noncoding or coding strand was identical at pH 7.5 or 5.5 in the presence of either magnesium or calcium (lanes 2–5). Treatment of reaction mixtures with 0.4 M NaCl prior to the addition of SDS abolished cleavage (Figure 5B, lanes 2–5). Since salt reversibility of DNA cleavage is a characteristic of topoisomerases (Liu et al., 1983; Osheroff & Zechiedrich, 1987), this result demonstrates that the observed cleavage was due to the action of the type II enzyme. The data presented in Figure 5A (lanes 2–4) show that under a variety of conditions in which topoisomerase II mediates high levels of DNA nicking (i.e., calcium at pH 5.5 or 7.5; magnesium at pH 5.5) (see Figure 4), the enzyme displayed a marked preference for cleavage at the noncoding over the coding strand. A similar result was found for the site-specific cleavage mediated by *Tetrahymena* and calf thymus type II enzymes (not shown). In the presence of magnesium at pH 7.5 (lane 5), the enzyme's preference for the noncoding strand appeared to be less pronounced. This is due primarily to the fact that topoisomerase II generates predominantly double-stranded, rather than single-stranded, breaks under these conditions (Zechiedrich et al., 1989) (see Figure 4).

Since electrophoresis on a denaturing gel converts duplex DNA fragments to single-stranded molecules, this system is unable to distinguish between cleavage bands which result from a nick or a double-stranded break. Therefore, reaction products were subjected to electrophoresis on a native polyacrylamide gel (Figure 6A). Due to the nondenaturing character of this gel system, fragments which contain an enzyme-mediated nick comigrate with the uncleaved 365 bp *Bam*HI–*Hin*dIII molecule, while the two fragments produced by double-stranded cleavage at the specific rDNA site migrate with sizes of 222 and 143 bp, respectively. As seen in Figure 6A, the highest levels of double-stranded breaks were observed at pH 7.5 (lanes 4 and 6). Moreover, at either pH employed, more double-stranded breaks were generated in reactions which contained calcium rather than magnesium (compare lane 3 to 5 and lane 4 to 6). Thus, the relative amounts of double-stranded cleavage at the specific rDNA site [Ca^{2+} (pH 7.5) > Mg^{2+} (pH 7.5) > Ca^{2+} (pH 5.5) > Mg^{2+} (pH 5.5)] were comparable to those found when negatively supercoiled pBR322 plasmid DNA was used as a substrate (see Figure 4).

Following removal of the doubly cleaved DNA background, it became possible to quantitate the preference of topoisomerase II for the noncoding strand of the rDNA site. To this end, the 365 bp bands containing uncleaved and nicked molecules were excised from lanes 3–6 of the native gel (Figure 6A). DNA was eluted and subsequently applied to a denaturing polyacrylamide gel. Results are shown in Figure 6B. As determined by densitometric scanning, *Drosophila* topoisomerase II displayed a ~10-fold preference for the noncoding over the coding strand in calcium at pH 5.5 (lane 3). In calcium at pH 7.5 (lane 4) or in magnesium at pH 5.5 (lane 5), the enzyme still displayed a strand specificity, although its preference (3–4-fold) for the noncoding strand was somewhat diminished. Levels of nicking in magnesium at pH 7.5 (lane 6) were too low to be detected on the autoradiogram shown in Figure 6B. Densitometric scanning of a stronger exposure of the autoradiogram, however, revealed a preference for the noncoding strand.

Religation of Cleaved DNA. The preference of topoisomerase II for the noncoding over the coding strand of the rDNA site may result from an enhanced forward rate of DNA cleavage or a decreased rate of religation. As the cleavage assays described above are at equilibrium, they cannot address this point. Therefore, a pre-steady-state DNA religation assay (Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989) was

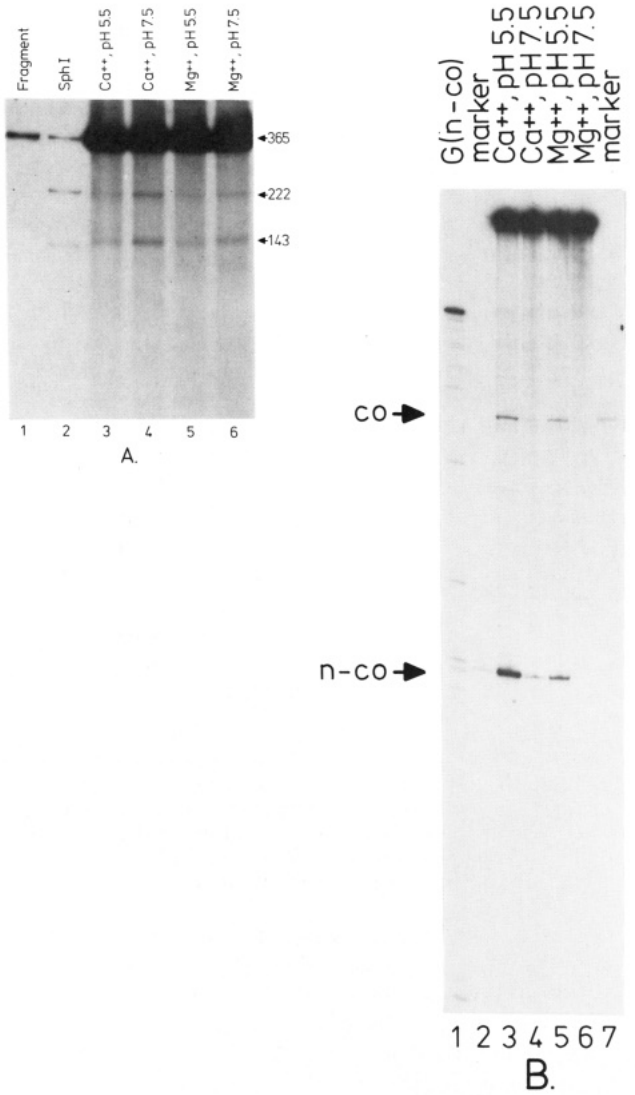

FIGURE 6: DNA strand specificity of topoisomerase II mediated cleavage at the rDNA site. (A) Topoisomerase II was incubated with the double-end-labeled *Bam*HI–*Hin*dIII fragment and processed as described in the legend of Figure 5A, except that cleavage products were analyzed on a 10% native polyacrylamide gel (lanes 3–6). Lane 1, untreated *Bam*HI–*Hin*dIII fragment; lane 2, *Sph*I digestion of the fragment. The numbers at the right give the sizes of DNA fragments in base pairs. (B) The 365 bp band was excised from lanes 3–6 on the native gel shown in (A). The DNA was eluted as described under Experimental Procedures and analyzed for single-stranded cleavage on a 6% denaturing polyacrylamide gel (lanes 3–6). DNA from the 143 bp band (lane 2) or the 222 bp band (lane 7) was purified from the sixth lane of Figure 6A and used as markers. Lane 1 shows the Maxam–Gilbert G reaction of the 5′-single-end-labeled *Xba*I–*Hin*dIII fragment (i.e., noncoding strand). Arrows indicate the cleavage positions on the two strands.

employed in order to determine the relative rates of religation of the noncoding and coding strands. The religation assay takes advantage of the finding that EDTA can be used to trap a kinetically competent covalent topoisomerase II–DNA cleavage complex which was formed in the presence of calcium. Following an increase in ionic strength to inhibit cleavage during the course of religation (Liu et al., 1983; Osheroff & Zechiedrich, 1987) (see Figure 5B), the enzyme-mediated rejoining of the cleaved DNA can be promoted by the addition of magnesium (Osheroff & Zechiedrich, 1987).

Topoisomerase II–DNA cleavage complexes were prepared for the religation assay in calcium at pH 7.5. Religation was started by the addition of magnesium at time zero. Samples were removed at 2–5-s intervals and analyzed on both a denaturing (Figure 7A) and a native (Figure 7B) polyacrylamide gel. Initial samples contained a mixture of doubly and singly cleaved DNAs. Within 2 s, double-stranded cleaved molecules had disappeared (Figure 7B), leaving only nicked molecules. This is consistent with the previous report that the enzyme rejoins the first DNA strand considerably faster than it does the second (Zechiedrich et al., 1989). Levels of residual DNA cleavage at time points which contained only nicked DNAs ($\geq$2 s) were quantitated by densitometric scanning of the denaturing gel shown in Figure 7A. A semilogarithmic plot of the data is shown in Figure 7C. While the absolute level of residual cleavage at the noncoding strand was approximately 4-fold greater than that at the coding strand, relative first-order rates of religation were similar. Therefore, under the conditions employed for this study, the DNA strand specificity of *Drosophila* topoisomerase II appears to be embodied in its forward cleavage rather than religation reaction.

Discussion

A strong recognition site for DNA cleavage by eukaryotic topoisomerase II has been isolated from the promoter region of the extrachromosomal rRNA genes of *T. thermophila*. This site incorporates many of the characteristics previously described for topoisomerase II recognition sites (Sander & Hsieh, 1983, 1985; Liu et al., 1983), including a staggered double-stranded break with a protruding four-base 5′ overhang and a lack of dyad symmetry around the point of cleavage.

The nucleotide sequence of the rDNA cleavage site shows no homology to a proposed consensus recognition sequence for *Drosophila* topoisomerase II (Sander & Hsieh, 1985) (Figure 8). Indeed, sequence identity is observed at only 2 of 11 positions where the consensus contains preferred bases. The rDNA site is more analogous to a cleavage site for the *Drosophila* enzyme previously identified in the bacterial plasmid pBR322 (Sander & Hsieh, 1983) (Figure 8). The 2 sequences are identical in 10 out of 16 positions overall, and 5 out of 5 positions immediately flanking the point of cleavage. This finding suggests that eukaryotic topoisomerase II might operate at several classes of recognition sequences.

To date, the structural and enzymatic properties of all eukaryotic type II topoisomerases examined appear to be similar (Wang, 1985; Vosberg, 1985; Maxwell & Gellert, 1986; Osheroff, 1989). The finding that type II topoisomerases from widely divergent eukaryotic species (*Drosophila*, calf thymus, and *Tetrahymena*) all cleave the rDNA site at the same nucleotide position suggests that these enzymes moreover display the same DNA sequence specificity.

In our study of the DNA strand specificity of topoisomerase II, we made use of the previous findings that (1) nicked (i.e., singly cleaved) DNA is an intermediate in the double-stranded cleavage reaction of topoisomerase II and (2) the enzyme's proclivity to nick DNA can be varied depending upon the reaction conditions employed (Zechiedrich et al., 1989). By quantitating the relative ratios of nicking at the noncoding and coding strands, it was possible to characterize the strand specificity of topoisomerase II.

Levels of enzyme-mediated nicking were modulated by exchanging calcium for magnesium and altering the pH of reaction mixtures. The highest amount of nicked rDNA products was generated in calcium-containing reactions at pH 5.5. Intermediate levels of nicking were observed in calcium at pH 7.5 or in magnesium at pH 5.5, while relatively low levels of nicked products were produced in the presence of magnesium at pH 7.5. Under all conditions, DNA nicking at the rDNA site took place with the hallmarks of the enzyme's double-stranded DNA cleavage reaction, including the covalent

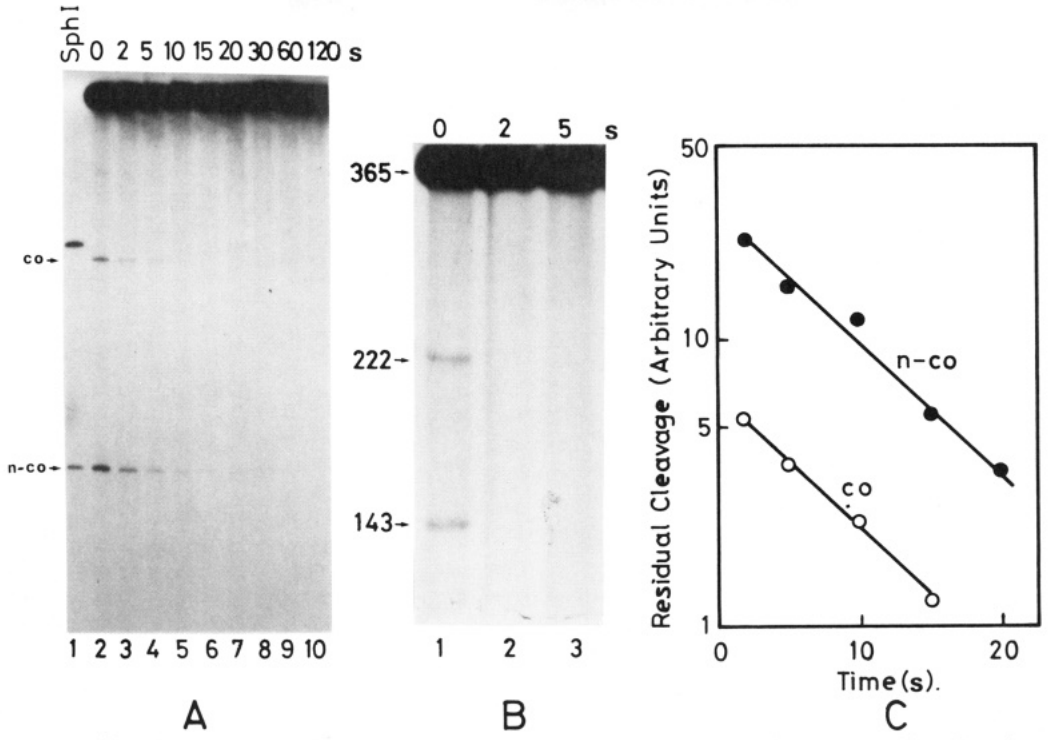

FIGURE 7: Time course of topoisomerase II mediated religation of cleaved DNA. Reaction products were subjected to electrophoresis on a 6% denaturing polyacrylamide gel (A) or a 10% native polyacrylamide gel (B) at the indicated time points following the onset of religation. *Sph*I digestion of the double-end-labeled *Bam*HI–*Hin*dIII fragment is shown in lane 1 (A). (C) Semilogarithmic plot of residual DNA cleavage versus time for the topoisomerase II mediated religation of the rDNA site. Levels of residual DNA cleavage were determined by densitometric scanning of the autoradiogram shown in (A) and are given in arbitrary integration units. Reactions were carried out as described under Experimental Procedures. Results for the noncoding (n-co, ●) and coding (co, ○) strands are shown. Plots represent the average of two experiments.

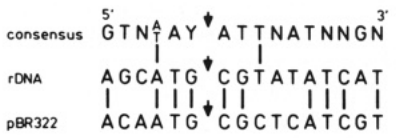

FIGURE 8: Comparison of the nucleotide sequence of the rDNA cleavage site with those of a proposed consensus sequence for *Drosophila* topoisomerase II and a strong recognition site for the *Drosophila* enzyme found in plasmid pBR322. The arrows denote the points of cleavage. Vertical bars denote positions of sequence identity. Y is for pyrimidine; N is for any base.

linkage of topoisomerase II to the 5′ termini of the cleaved DNA (not shown) and salt reversibility. Moreover, the nucleotide positions of enzyme-generated nicks were identical with those of double-stranded cleavage.

As determined in the above system, topoisomerase II displayed strand specificity in its interaction with the rDNA site. Under conditions which promoted considerably levels of topoisomerase II mediated nicking, the enzyme showed a marked preference for the noncoding strand, cleaving it up to 10-fold more efficiently than it did the coding strand. Similar results were obtained using *Drosophila*, *Tetrahymena*, or calf thymus topoisomerase II, suggesting that strand discrimination is an intrinsic property of all eukaryotic type II enzymes.

The relative first-order rates of religation of the noncoding and coding strands were similar. Thus, it appears that strand specificity is a property of the enzyme's forward cleavage rather than religation reaction. The structural features of the topoisomerase II cleavage site which lead to the enzyme's strand specificity have yet to be determined.

DNA topoisomerase II is an essential enzyme which is involved in many fundamental cellular processes (Nelson et al., 1986; Brill et al., 1987; Yang et al., 1987; DiNardo et al., 1984; Earnshaw & Heck, 1985; Gasser et al., 1986). Central to its physiological functions is its ability to create transient double-stranded breaks at specific sites in the genome (Udvardy et al., 1986; Rowe et al., 1986; Yang et al., 1985). Recently, topoisomerase II has been shown to be the target for a number of clinically relevant chemotherapeutic drugs (Ross, 1985; Zwelling, 1985; Glisson & Ross, 1987). The antineoplastic activities of these agents correlate with their abilities to interfere with the enzyme's DNA cleavage–religation equilibrium (Long et al., 1984). In addition, these agents also impair the enzyme's ability to recognize its DNA sites of interaction. When drugs are present, the nucleotide specificity of topoisomerase II becomes less stringent, and the enzyme cleaves DNA at a variety of novel or poorly utilized sites (Glisson & Ross, 1987). Although correlations have yet to be drawn, this latter effect is likely to contribute to the cytotoxic effects of these drugs. Clearly, before the metabolic functions of topoisomerase II or the chemotherapeutic activities of the above drugs can be fully understood, the mechanism by which the enzyme determines its genomic sites of action must be delineated. The results of this study indicate that topoisomerase II not only recognizes the nucleotide sequence of its DNA cleavage site but also is able to discriminate between the two complementary strands of the double helix as well.

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