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Eukaryotic topoisomerases recognize nucleic acid topology by preferentially interacting with DNA crossovers

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Eukaryotic topoisomerases recognize DNA topology and preferentially react with positively or negatively supercoiled molecules over relaxed substrates. To elucidate the mechanism of this recognition, we examined the interaction of topoisomerases with DNA by electron microscopy. Under all conditions employed, ~90% of the bound type I or II enzyme was observed at points of helix-helix juxtaposition on negatively supercoiled plasmids which contained as few as four crossovers. Recognition was independent of torsional stress, as enzyme molecules were also found at crossovers on linear DNA. Since juxtaposed helices are more prevalent in supercoiled compared with relaxed nucleic acids, we propose that eukaryotic topoisomerases I and II recognize underwound or overwound substrates by interacting preferentially with DNA crossovers. This may represent a general mechanism for the recognition of DNA topology by proteins.

Key words: DNA cleavage/DNA crossovers/DNA topology/topoisomerase I/topoisomerase II

Introduction

Virtually every facet of nucleic acid physiology is influenced by the topological state of DNA (Wang, 1985). For example, the underwinding and overwinding of the double helix profoundly alters the initiation and elongation of both DNA replication and transcription (Fuller *et al.*, 1981; Harland *et al.*, 1983; Wang, 1985; Baker *et al.*, 1986; Kelly, 1988; Tabuchi and Hirose, 1988). Moreover, intra- and intermolecular knots must be untangled in order to resolve recombination products and unlink daughter chromosomes (Sundin and Varshavsky, 1981; Spengler *et al.*, 1985; Wang, 1985; Weaver *et al.*, 1985; Wasserman and Cozzarelli, 1986). Clearly, the cell's ability to regulate the topological state of DNA is imperative for its viability.

Physiologically, DNA topology is modulated by two classes of ubiquitous enzymes, the type I and type II topoisomerases. Topoisomerase I can relieve torsional constraints in DNA by passing a single strand of DNA through a transient nick made in the complementary strand (Wang, 1985; Osheroff, 1989). Although the type I enzyme is not required for cell survival (Thrash *et al.*, 1984, 1985; Uemura and Yanagida, 1984), it plays important roles in DNA replication (Goto and Wang, 1985; Snapka, 1986; Brill *et al.*, 1987; Yang *et al.*, 1987) and transcription (Bonven *et al.*, 1985; Muller *et al.*, 1985; Gilmour *et al.*, 1986; Brill *et al.*, 1987; Garg *et al.*, 1987). Topoisomerase II can relieve both torsional and interlocking constraints in double-stranded nucleic acids by passing an intact helix through a transient double-stranded break made in a second helix (Wang, 1985; Osheroff, 1989). In contrast to the type I enzyme, topoisomerase II is essential to the eukaryotic cell (Goto and Wang, 1984; DiNardo *et al.*, 1984; Uemura and Yanagida, 1984; Holm *et al.*, 1985). It is required for chromosome segregation (Uemura and Yanagida, 1984, 1986; DiNardo *et al.*, 1984; Holm *et al.*, 1985; Uemura *et al.*, 1987) and the maintenance of proper chromosome structure (Berrios *et al.*, 1985; Earnshaw and Heck, 1985; Earnshaw *et al.*, 1985; Gasser and Laemmli, 1986; Gasser *et al.*, 1986) and may play roles in DNA replication (Wang, 1985; Snapka, 1986; Yang *et al.*, 1987; Brill *et al.*, 1987; Osheroff, 1989) and recombination (Bae *et al.*, 1988; Dillehay *et al.*, 1989; Rose *et al.*, 1990).

A critical mechanistic feature of most enzymes is their ability to distinguish between the substrates and products of their reactions. In this regard, DNA topoisomerases are no exception. As determined by binding and kinetic assays, both eukaryotic topoisomerase I (Muller, 1985; Camilloni *et al.*, 1988, 1989) and II (Osheroff and Brutlag, 1983; Osheroff *et al.*, 1983; Osheroff, 1986, 1987) display a 3- to 5-fold higher affinity for negatively supercoiled substrates over relaxed DNA. Since the chemical structures of supercoiled and covalently closed relaxed DNA molecules are identical, topoisomerases must be able to discern substrate from product purely on the basis of topological differences. This raises the question of how topoisomerases are able to determine the topological state of DNA. Features unique to negatively supercoiled (underwound) molecules such as increased single-stranded character, cruciforms, or Z-DNA (Wells, 1988) are not likely to be recognized by these enzymes, since (i) positively and negatively supercoiled DNA are equivalent substrates for eukaryotic topoisomerases (Champoux and Dulbecco, 1972; Goto and Wang, 1982; Benedetti *et al.*, 1983; Osheroff *et al.*, 1983; Schomburg and Grosse, 1986), and (ii) the overwinding associated with positively supercoiled DNA destabilizes the above structures. The only obvious features that underwound and overwound nucleic acids have in common are regions of helix-helix juxtaposition (DNA crossovers). On the basis of this observation, it was suggested that *Drosophila* topoisomerase II recognizes superhelicity by preferentially interacting with DNA crossovers (Osheroff and Brutlag, 1983).

In order to test this hypothesis, the interaction of eukaryotic topoisomerases with DNA was examined by electron microscopy. Results of this study indicate that both the type I and type II enzymes recognize supercoiled DNA (at least in part) by interacting preferentially with crossover points on DNA. This may represent a general mechanism by which proteins can recognize the topological state of nucleic acids.

Results

Topoisomerase II recognizes DNA topology independent from its ability to distinguish nucleic acid sequence

Eukaryotic topoisomerases recognize two properties of their DNA substrates. On the one hand, the type I and II enzymes can discern the topological state of DNA. This is demonstrated by the results of binding and kinetic assays in which both enzymes displayed a 3- to 5-fold higher affinity for negatively supercoiled molecules than for relaxed or linear substrates (Osheroff and Brutlag, 1983; Osheroff *et al.*, 1983; Osheroff, 1986, 1987; Camilloni *et al.*, 1988, 1989). In addition, topoisomerase I and II relax positively supercoiled plasmids at rates which are comparable to those for negatively supercoiled molecules (Champoux and Dulbecco, 1972; Goto and Wang, 1982; Benedetti *et al.*, 1983; Osheroff *et al.*, 1983; Schomburg and Grosse, 1986). Thus, the eukaryotic enzymes can distinguish DNA topology, but cannot discern the directionality of superhelical twisting. On the other hand, topoisomerase I and II recognize the primary structure of DNA and cleave the nucleic acid backbone at preferred sites (Wang, 1985; Osheroff, 1989). As determined by binding, kinetic, and footprinting studies, sites of DNA cleavage mapped *in vitro* correspond to sites of topoisomerase catalytic action (Busk *et al.*, 1987; Sander *et al.*, 1987; Lee *et al.*, 1989).

Previous studies indicate that topoisomerase I recognizes DNA topology and primary structure by independent mechanisms (Camilloni *et al.*, 1989). Although the effects of DNA topology on topoisomerase II-mediated DNA binding, strand passage and ATP hydrolysis have been described, the relationship between the type II enzyme's recognition of DNA topology as opposed to its recognition of nucleic acid sequence has yet to be determined. Two experiments were carried out in order to characterize this relationship. First, the effect of superhelical density on the efficiency of topoisomerase II-mediated DNA cleavage was analyzed. Assays were carried out in the absence of ATP. Therefore, levels of DNA breakage reflect the enzyme's cleavage/religation equilibrium which is established prior to DNA strand passage (Osheroff, 1989). As seen in Figure 1, over a wide range of enzyme concentration, ~3 times more cleavage was observed with negatively supercoiled pBR322 DNA than with relaxed plasmid substrate.

Second, the effect of superhelical density on the recognition of DNA cleavage sites by topoisomerase II was determined. Since the enzyme cleaves underwound DNA more efficiently than relaxed molecules, it is possible that torsional stress either (i) opens new classes of DNA cleavage sites for topoisomerase II or (ii) alters the enzyme's utilization of existing sites. However, at the resolution of agarose gel electrophoresis, the enzyme cleaved negatively supercoiled and relaxed pBR322 DNA at identical sites (Figure 2). Even the relative utilization of cleavage sites on the two substrates was the same. The topoisomerase II-mediated DNA cleavage products displayed in Figure 2 were generated in calcium-containing buffers. Consistent with previous reports (Sander and Hsieh, 1983; Osheroff and Zechiedrich, 1987; Osheroff, 1987), no cleavage was observed in the absence of a divalent cation (Figure 2, control lane). Although the enzyme cleaves DNA somewhat more efficiently in calcium- than in magnesium-containing buffers, it utilizes the same nucleic acid recognition sequences with either

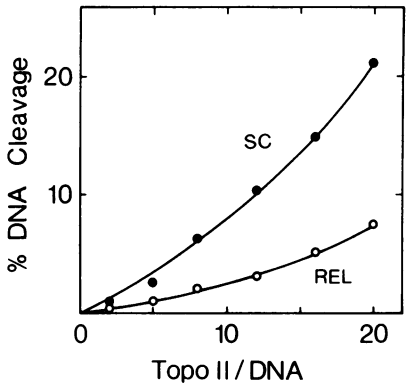

Fig. 1. Topoisomerase II preferentially cleaves negatively supercoiled substrate over relaxed plasmid DNA. Cleavage reactions contained 0–100 nM *Drosophila* topoisomerase II and 5 nM negatively supercoiled or relaxed pBR322 plasmid DNA in the presence of 5 mM $MgCl_2$.

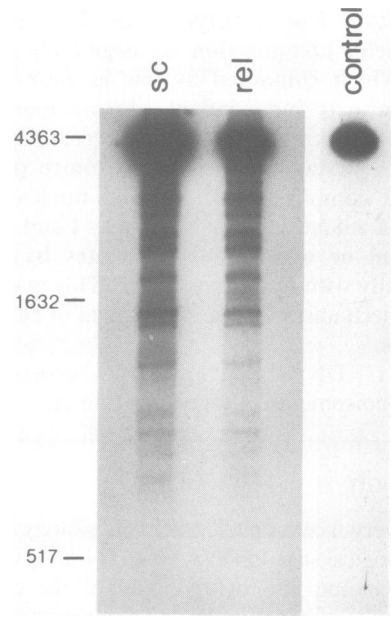

Fig. 2. Sites of DNA cleaved by topoisomerase II are independent of the topological state of the nucleic acid substrate. Topoisomerase II-mediated DNA cleavage maps of either negatively supercoiled (sc) or relaxed (rel) plasmid substrate were generated in the presence of calcium as described in Materials and methods. A control reaction which contained enzyme and negatively supercoiled DNA but no divalent cation is also shown. Molecular weight markers are indicated.

divalent cation (Osheroff and Zechiedrich, 1987; Andersen *et al.*, 1989; Zechiedrich *et al.*, 1989). As expected, results identical to those in Figure 2 were obtained when magnesium replaced calcium in cleavage reactions (not shown). In addition to the above, sites of topoisomerase II-mediated DNA cleavage on positively supercoiled pBR322 molecules were also mapped and were found to be identical to those for negatively supercoiled or relaxed substrates (E.L. Zechiedrich and N.Osheroff, unpublished results). These results indicate that the enzyme's ability to recognize the topological state of DNA is independent of its ability to recognize nucleic acid sequence. Thus, while DNA topology regulates levels of topoisomerase activity, nucleic acid sequence dictates the specificity of both the type I and II enzymes.

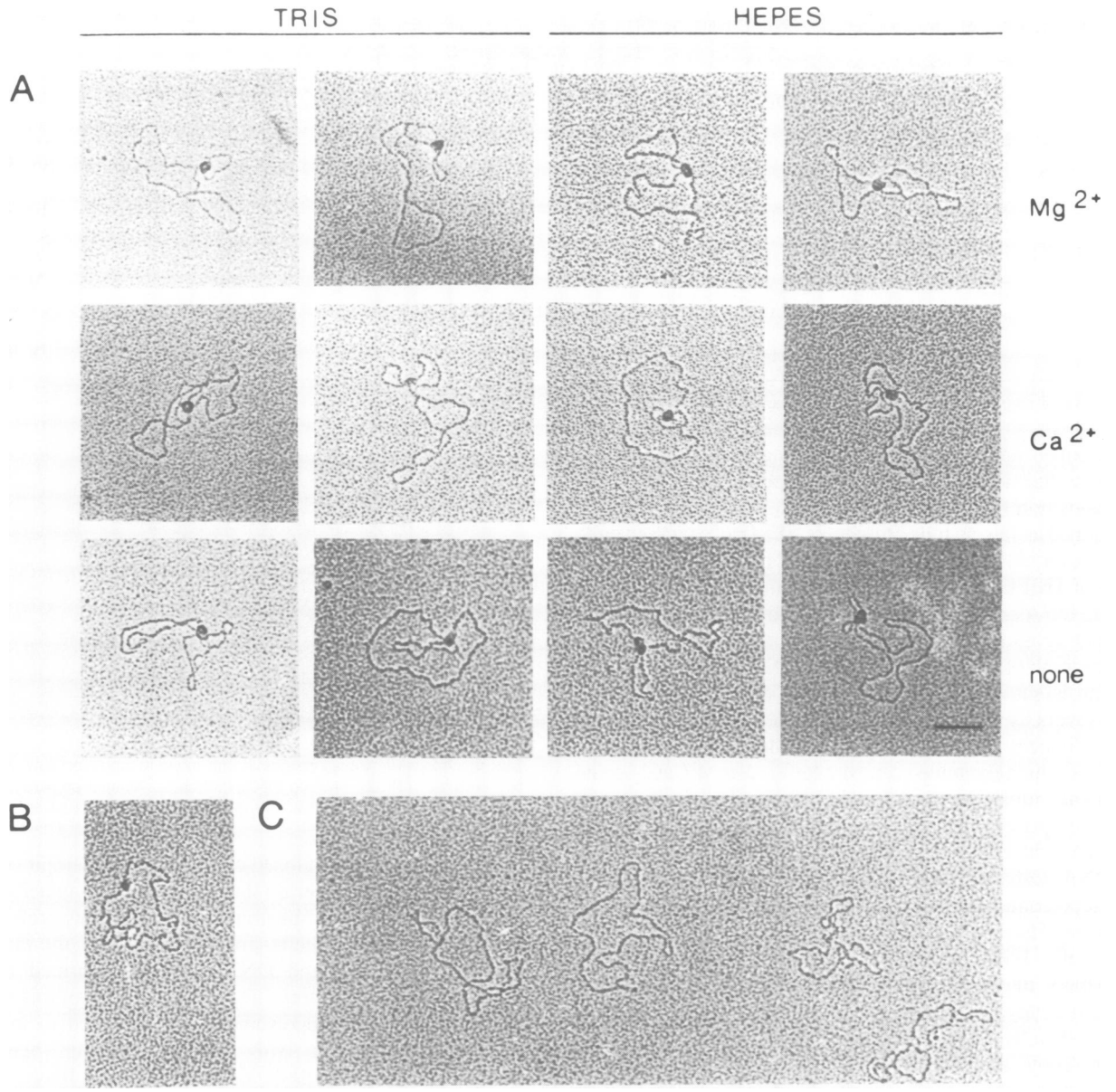

Fig. 3. Recognition of DNA helix–helix juxtaposition points in circular plasmids by topoisomerase II. Samples were prepared for electron microscopy as described by Griffith and Christiansen (1978). (**A**) *Drosophila* topoisomerase II was incubated with partially supercoiled plasmid substrate in Tris (left two columns) or HEPES (right two columns) buffer in the presence of either 5 mM $MgCl_2$ (top row), 5 mM $CaCl_2$ (middle row) or no divalent cation (bottom row). A representative micrograph of the calf thymus type II enzyme bound to plasmid DNA in the presence of HEPES and 5 mM $MgCl_2$ is shown in the top right panel. The bar represents 1000 Å. (**B**) A representative micrograph of *Drosophila* topoisomerase II bound to DNA at a non-crossover region is shown. (**C**) Partially negatively supercoiled pBR322 plasmid substrate in the absence of topoisomerase is shown.

Topoisomerase II recognizes DNA topology by preferentially interacting with points of helix–helix juxtaposition

As described above, eukaryotic topoisomerase II can discriminate supercoiled from relaxed DNA molecules (see Figure 1), but cannot distinguish the directionality of superhelical twisting. The only common structural features which are prevalent in both underwound and overwound DNA but not in relaxed molecules are points of helix–helix juxtaposition (i.e., DNA crossovers or nodes) which are generated by torsional constraints. Therefore, the ability of *Drosophila* topoisomerase II to recognize crossovers on circular DNA substrates was determined by electron microscopy. It should be noted that all of the studies described below were carried out in the absence of ATP, the high energy cofactor which is required for DNA strand passage by the type II enzyme (Wang, 1985; Osheroff, 1989).

Because of the intertwined nature of superhelically twisted DNA, it is difficult to localize specific interactions between highly supercoiled nucleic acids and proteins by electron microscopy. To overcome this problem, plasmid substrates which contained only 0–5 negative superhelical twists were generated by the procedure of Singleton and Wells (1982) and were employed for this study. Samples were spread for electron microscopy by the technique of Griffith and Christiansen (1978). A typical field of partially supercoiled plasmids in the absence of topoisomerase is shown in Figure 3C.

When topoisomerase II was incubated with partially

Table I. Percentage of eukaryotic topoisomerase II bound to DNA crossovers in circular plasmids[a]

Enzyme source	Reaction conditions		DNA crossovers/plasmid			
	Buffer	Divalent cation	1	2	3	≥4
Drosophila	HEPES	Mg^{2+}	72% (36)	77% (51)	89% (36)	97% (33)
		Ca^{2+}	31% (126)	45% (119)	50% (50)	84% (94)
		None	75% (92)	91% (68)	93% (54)	100% (21)
Drosophila	Tris	Mg^{2+}	38% (189)	72% (218)	75% (107)	89% (71)
		Ca^{2+}	83% (47)	98% (42)	100% (31)	95% (18)
		None	39% (145)	39% (137)	45% (76)	79% (112)
Calf	HEPES	Mg^{2+}	54% (28)	57% (24)	74% (33)	81% (84)

[a]Numbers in parentheses represent the total number of topoisomerase II–pBR322 DNA complexes scored under each set of conditions.

supercoiled substrates and analyzed by electron microscopy, the enzyme was found primarily at points of helix–helix juxtaposition (Figure 3A and Table I). On plasmids which contained as few as four crossovers, the *Drosophila* enzyme was observed at DNA nodes ~90% of the time in either Tris or HEPES buffer. Even on plasmid molecules with a single DNA crossover, bound topoisomerase II was located at that crossover ~50% of the time. (A representative micrograph of the enzyme bound to DNA at a non-crossover region is shown in Figure 3B.) The recognition of DNA crossovers by topoisomerase II did not require the presence of a divalent cation, as similar results were obtained in buffers which contained 5 mM $MgCl_2$, 5 mM $CaCl_2$ or no divalent cation (Figure 3A and Table I). This is consistent with the previous observation that divalent cations do not influence the enzyme's ability to discern the topological state of DNA (Osheroff, 1987). Since a divalent cation is required for topoisomerase II-mediated DNA cleavage and strand passage (Hsieh and Brutlag, 1980; Osheroff *et al.*, 1983; Osheroff, 1987), this finding also demonstrates that the enzyme's interaction with nodes precedes both of these critical catalytic events.

As detailed under Materials and methods, electron microscopy samples were prepared by three different techniques. Thus, the localization of topoisomerase II to regions of helix–helix juxtaposition is not likely to be influenced by the microscopy protocols. Relevant to this point, similar protocols have been employed to examine protein–DNA interactions with histones, DNA polymerase, or RNA polymerase (discussed in Griffith and Christiansen, 1978), and none of the above was observed at DNA crossovers. In addition, the interaction of topoisomerase II with crossovers did not depend on glutaraldehyde fixation, as the enzyme was also localized at DNA nodes in the absence of any fixative (data not shown).

Finally, preferential interaction with DNA crossovers was not exclusive to the type II enzyme from *Drosophila*. In a series of experiments with calf thymus topoisomerase II, the mammalian enzyme was also found at regions of helix–helix juxtaposition ~80% of the time on plasmids which contained 4 nodes (Table I and Figure 3A, top right panel).

Recognition of DNA crossovers by topoisomerase II does not require that the DNA be under torsional stress

The intramolecular DNA crossovers described in the previous section resulted from torsional stress (i.e., underwinding) in the closed circular plasmid employed. If the ability of topoisomerase II to recognize both underwound and overwound DNA substrates results from its preference for points of helix–helix juxtaposition, then the enzyme should be able to bind DNA crossovers formed in the absence of torsional stress. During the course of this work, some molecules of topoisomerase II appeared to interact simultaneously with two separate circular plasmids (Figure 4). This suggests that the enzyme may indeed recognize DNA nodes which are not induced by torsional constraints. To confirm this observation, the interaction between topoisomerase II and linear DNA fragments was examined.

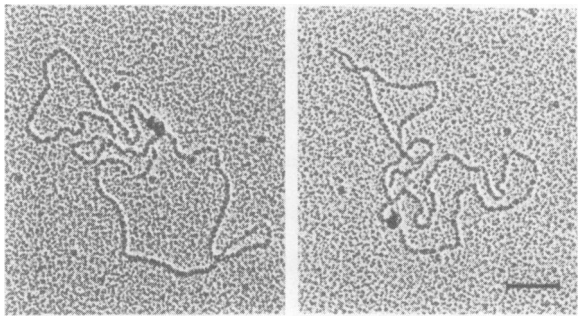

Fig. 4. *Drosophila* topoisomerase II can interact simultaneously with two separate pBR322 plasmid DNA molecules. Sample preparation was as described in Materials and methods. The bar represents 1000Å.

As seen in Figure 5, topoisomerase II was observed at DNA crossovers on linear nucleic acid molecules. The *Drosophila* enzyme was localized to nodes in ~35% of the 286 complexes which were scored. Similar results were found when calf thymus topoisomerase II was employed (Figure 5, bottom right panel). The mammalian enzyme was seen at crossovers in ~31% of the 130 complexes scored. While these percentages are somewhat lower than those generated with partially supercoiled circular plasmids the incidence of crossovers observed with linear DNA molecules in the absence of topoisomerase II was only 2–3%. Moreover, levels of enzyme bound to nodes in linear DNA fragments were in a range similar to those found for its interaction with nodes on circular molecules containing a single DNA crossover (~50%) (see Table I). Finally, of the 100 *Drosophila* topoisomerase II molecules visualized at DNA nodes, 64 were bound to two separate DNA fragments (Figure 5).

The topoisomerase II–linear DNA complexes shown in

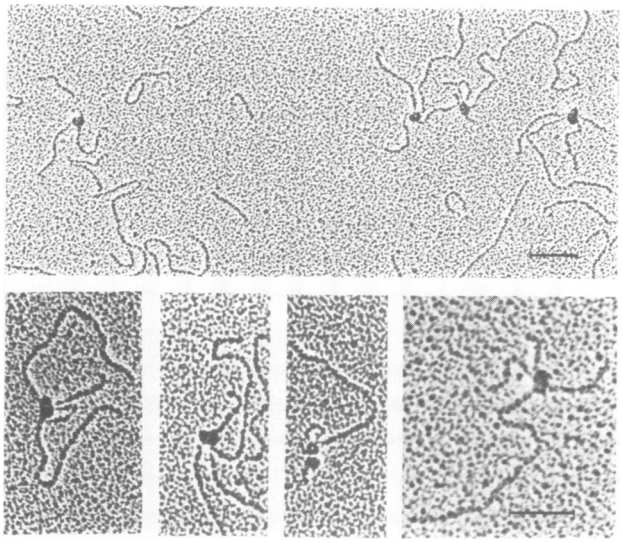

Fig. 5. Topoisomerase II binds DNA crossovers on linear substrates. Purified fragments of pBR322 DNA, 376, 753, 1540 and 1693 bp in length, were incubated with *Drosophila* topoisomerase II in the presence of Tris and 5 mM $MgCl_2$ and prepared for electron microscopy as described in Materials and methods. A typical field is shown in the top panel. Topoisomerase II–linear DNA complexes are shown at a higher magnification in the bottom panels. A representative micrograph of the calf thymus type II enzyme bound to linear DNA is shown in the bottom right panel bar. The bar represents 1000 Å.

Figure 5 were generated following a 6 min binding incubation. To characterize the effect of incubation time on the recognition of DNA crossovers by the *Drosophila* enzyme, a time course of binding was examined (Figure 6). Approximately 300 enzyme–DNA complexes were scored for each time point. Under the conditions employed, levels of topoisomerase II bound at crossovers remained constant at ~32% between 10 s and 6 min. Furthermore, the ratio of enzyme found at inter- versus intramolecular DNA crossovers (~2:1) was also constant over the time course examined. These results demonstrate that under the conditions employed, topoisomerase II binds regions of helix–helix juxtaposition in an equilibrium fashion. Therefore, as would be expected of an enzyme that recognizes positively as well as negatively supercoiled substrates, topoisomerase II interacts with DNA crossovers which are not constrained by torsional stress.

Eukaryotic topoisomerase I also recognizes DNA crossovers

Although topoisomerase I discerns DNA topology with a specificity similar to that of topoisomerase II, the two enzymes catalyze alterations in topological structure by different mechanisms. The type II enzyme acts by passing an intact double helix of DNA through a transient break made in a second double helix (Wang, 1985; Osheroff, 1989). This catalytic mechanism mandates that topoisomerase II interact simultaneously with two DNA helices at least at the time of strand passage. In contrast, the type I enzyme acts by passing a single strand of DNA through a transient nick made in the complementary strand (Wang, 1985; Osheroff, 1989). This catalytic mechanism does not require that topoisomerase I interact with more than one double helix at any point in its reaction cycle.

In spite of its mechanistic differences from topoisomerase

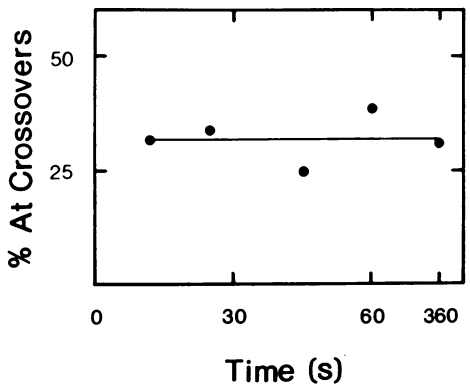

Fig. 6. Time course of *Drosophila* topoisomerase II recognition of DNA crossovers in linear substrates. Enzyme–DNA complexes were generated as described in Figure 5. Approximately 300 complexes were scored for each time point.

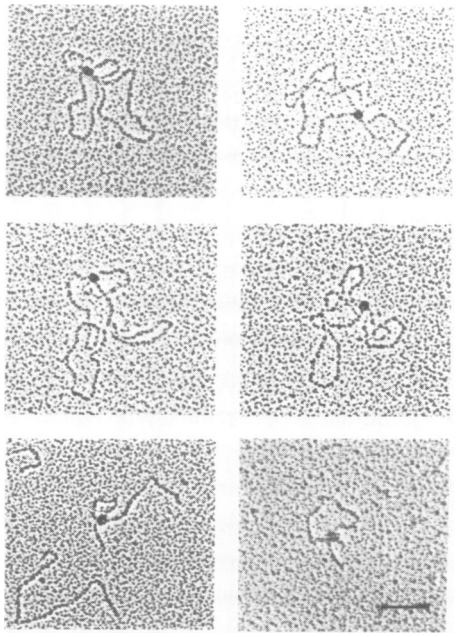

Fig. 7. Electron micrographs of eukaryotic topoisomerase I bound at DNA crossovers on circular and linear DNA substrates. Calf thymus topoisomerase I was incubated with circular (top two rows) or linear (bottom row) DNA substrates as described in Materials and methods. The bar represents 1000 Å.

II, the preference of topoisomerase I for underwound or overwound DNA over relaxed molecules suggests that it may also discern nucleic acid topology by binding to points of helix–helix juxtaposition. To this end, the interaction of calf thymus topoisomerase I with covalently closed circular pBR322 plasmid DNA was characterized. Typical electron micrographs are shown in Figure 7 (top two rows). As found for the type II enzyme, eukaryotic topoisomerase I was bound to DNA at crossovers. In plasmid molecules which contained 3 or 4 DNA nodes, the enzyme was found at crossovers in 100% or 97% of the complexes scored (36 or 59 complexes), respectively. These results indicate that eukaryotic topoisomerase I recognizes nucleic acid topology by interacting with DNA crossovers, even though its catalytic mechanism shows no apparent requirement for such an interaction. Therefore, the ability to bind points of

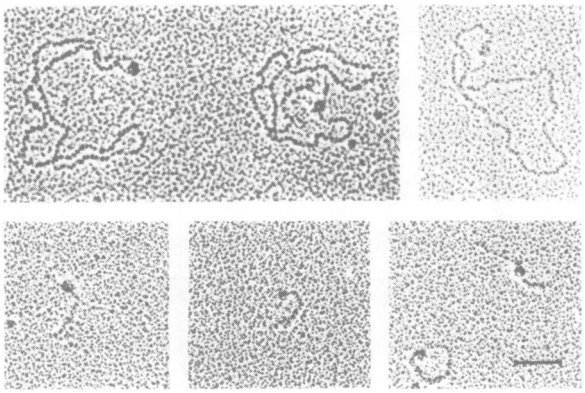

Fig. 8. Electron micrographs of ω protein bound to circular and linear DNA substrates. *E.coli* ω protein was incubated with circular (top row) or linear (bottom row) DNA substrates as described in Materials and methods. The bar represents 1000Å.

helix−helix juxtaposition may represent a general method for protein recognition of DNA topology.

To examine further the recognition of DNA nodes by eukaryotic topoisomerase I, the enzyme's interaction with linear fragments of pBR322 was also visualized (Figure 7, bottom row). Topoisomerase I was found at intramolecular DNA crossovers at the same level (13% of 110 complexes scored) as the type II enzyme (13% of 286 complexes scored). However, unlike the type II enzyme, topoisomerase I was never found at nodes generated by two separate DNA fragments. While these findings confirm that the eukaryotic type I enzyme recognizes points of helix−helix juxtaposition which are not torsionally constrained, they also point to mechanistic differences between the type I and II enzymes.

Prokaryotic topoisomerase I (ω protein) does not recognize DNA crossovers

Bacterial ω protein displays a specificity for DNA topology which is different from that of the eukaryotic type I or type II enzyme. Although ω protein interacts preferentially with negatively supercoiled over relaxed substrates, it shows no kinetic affinity for positively supercoiled (overwound) DNA (Wang, 1971; Kirkegaard and Wang, 1985). Unlike the eukaryotic topoisomerases, ω protein distinguishes DNA topology by binding to regions with single-stranded character stabilized by the underwinding of the double helix (Kirkegaard and Wang, 1985). This preference for single-stranded regions accounts for the enzyme's inability to interact with overwound DNA.

Because of the mode by which ω protein discerns DNA topology, it would not be expected to possess an intrinsic ability to recognize DNA crossovers. As shown in Figure 8, this is the case. On circular pBR322 plasmid molecules with 3 or 4 DNA crossovers, *Escherichia coli* ω protein was observed at nodes in only 20% or 12% of the complexes scored (55 or 84 complexes), respectively. This is in marked contrast to results with the mammalian type I enzyme, which under the same conditions was found at intramolecular crossovers >97% of the time. Similarly, ω protein was seen at crossovers on linear DNA in ~2.5% of the complexes scored (293 complexes) as compared to the ~13% observed with the calf thymus type I enzyme. The ω protein was never seen at intermolecular crossovers.

Discussion

The results of this study provide a mechanistic basis for the recognition of DNA topology by eukaryotic topoisomerases. As visualized by electron microscopy, both topoisomerase I and II bound DNA at points of helix−helix juxtaposition. Such DNA crossovers are more prevalent in torsionally stressed nucleic acids compared with relaxed molecules, irrespective of the directionality of torsional stress. Thus, we propose that the preference of these enzymes for crossovers accounts for their ability to distinguish the topological state of DNA.

Topoisomerase II cleaved negatively supercoiled DNA more efficiently than it did relaxed molecules. However, sites of nucleic acid hydrolysis as well as the relative utilization of sites were the same with both substrates. A similar result has been reported for topoisomerase I (Camilloni *et al.*, 1989). This indicates that the eukaryotic topoisomerases recognize DNA topology and discern nucleic acid sequence by independent mechanisms. Therefore, the abundance of DNA crossovers regulates the catalytic efficiency of topoisomerases I and II, but does not influence the nucleic acid specificity of either enzyme.

In contrast to the eukaryotic topoisomerases which remove supercoils from DNA substrates, the prokaryotic type II enzyme (gyrase) is able to introduce negative superhelical twists into nucleic acid molecules. Consequently, for its supercoiling reaction, gyrase preferentially interacts with relaxed or linear substrates over torsionally constrained molecules (Sugino and Cozzarelli, 1980; Higgins and Cozzarelli, 1982). A previous study revealed that gyrase was also found on crossovers at the base of DNA loops in relaxed pBR322 plasmids (Moore *et al.*, 1983). This localization most likely results from the enzyme's catalytic mechanism rather than from its ability to discern DNA topology. Gyrase induces superhelical twisting by specifically wrapping DNA around itself prior to strand passage (reviewed in Maxwell and Gellert, 1986). Thus, the enzyme probably creates the loops to which it is bound, and interacts more readily with relaxed or linear DNA molecules because of their greater flexibility.

Eukaryotic topoisomerase II acts by passing an intact DNA helix through a transient break made in a second helix. This enzymatic mechanism requires that topoisomerase II interact simultaneously with two DNA helices at least at the time of strand passage. The localization of topoisomerase II to DNA crossovers in the absence of a divalent cation or ATP indicates that the enzyme in fact binds juxtaposed helices prior to either its DNA cleavage or strand passage events. Although relationships between the two bound helices have yet to be explored, the most simplistic interpretation is that one represents the helix which is cleaved by the enzyme and the other represents the helix which is translocated through the break. Since topoisomerase II binds both helices prior to cleavage, it is tempting to speculate that the presence of the passing helix stimulates or is even required for the enzyme to cleave its DNA recognition site. Experiments are currently under way in the laboratory to address this point.

Although eukaryotic topoisomerase I discerns DNA topology with a specificity that is identical to that of the eukaryotic type II enzyme, its catalytic single-strand DNA passage reaction never requires it to interact with multiple DNA helices. Therefore, the type I enzyme may recognize

DNA nodes solely to discriminate supercoiled substrates from relaxed products.

An important question raised by this study is that of how topoisomerases find DNA crossovers. One possibility is that the enzymes recognize and bind directly to pre-existing crossovers in DNA. Another possibility is that they initially bind a single DNA helix, then search and subsequently find a second DNA helix. The latter possibility is supported by the observation that under equilibrium conditions, both topoisomerase I and II were able to interact with a single DNA helix. This was seen not only with linear DNA fragments, but with covalently closed relaxed circular plasmid molecules as well (data not shown). While eukaryotic topoisomerases I and II appear to bind two DNA helices in a sequential manner, the mechanisms by which they search for the second helix may differ. Since topoisomerase I was never found spanning intermolecular DNA crossovers, this suggests that the type I enzyme binds initially to a single DNA helix and finds its second helical segment primarily by a one dimensional search (von Hippel and Berg, 1989). Conversely, topoisomerase II was often found at intermolecular DNA nodes formed between linear or circular nucleic acid substrates. This latter observation precludes the possibility that the enzyme finds DNA crossovers by only a one dimensional process.

The data presented in this study establish a common teleological thread which connects the structural and catalytic functions of topoisomerase II in the eukaryotic cell. Structurally, the enzyme appears to play an important role in the maintenance of chromosome organization. It is a major polypeptide of the interphase nuclear matrix (Berrios *et al.*, 1985) and it is the most abundant protein component of the mitotic chromosome scaffold (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986). On the basis of its distribution pattern within the chromosome scaffold (Earnshaw and Heck, 1985) and its ability to bind, aggregate, and cleave matrix or scaffold associated regions of DNA (MAR or SAR sequences, respectively) (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Adachi *et al.*, 1989; Sperry *et al.*, 1989), topoisomerase II has been proposed to anchor chromatin loop domains *in vivo*. The present finding that the enzyme can simultaneously bind two separate DNA helices, even in the absence of the divalent cation and high energy cofactor necessary for its catalytic activity, may account for the ability of topoisomerase II to organize chromatin loops.

Catalytically, the essential function of topoisomerase II is the disentanglement of daughter chromosomes at the time of mitosis (Uemura and Yanagida, 1984, 1986; DiNardo *et al.*, 1984; Holm *et al.*, 1985; Uemura *et al.*, 1987). During this process, the enzyme must carry out strand passage of DNA helices from two distinct, but topologically intertwined chromosomes. The observation that topoisomerase II displays an affinity for points of helix–helix juxtaposition generated by intermolecular interactions may explain the enzyme's efficient resolution of mitotic chromosomes.

In summary, the abilities of eukaryotic topoisomerase I and II to discern the topological structure of DNA correlate with their preference for points of helix–helix juxtaposition in nucleic acid substrates. Therefore, we conclude that recognition of DNA crossovers may represent a general mechanism by which proteins can distinguish the topological state of DNA.

Materials and methods

Materials

Drosophila melanogaster topoisomerase II was purified from the nuclei of Kc tissue culture cells by the procedure of Shelton *et al.* (1983). Calf thymus topoisomerase II was isolated as described by Andersen *et al.* (1989) and was the generous gift of Dr P.S.Jensen and Dr O.Westergaard. Calf thymus topoisomerase I was purchased from Bethesda Research Laboratories. *E.coli* topoisomerase I (ω protein) was generously provided by Dr J.Kaguni. Negatively supercoiled pBR322 plasmid DNA was isolated from *E.coli* DHI by a Triton X-100 lysis procedure followed by double banding in cesium chloride–ethidium bromide gradients (Sambrook *et al.*, 1989). All chemicals employed were analytical reagent grade.

DNA cleavage assay

Cleavage reactions contained 0–100 nM *Drosophila* topoisomerase II and 5 nM negatively supercoiled or relaxed pBR322 plasmid DNA in a total of 20 μl of cleavage buffer [10 mM Tris (pH 7.9), 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA and 2.5% glycerol] that contained 5 mM $MgCl_2$. Samples were incubated at 30°C for 6 min. DNA cleavage products were trapped by the addition of 2 μl of 10% SDS followed by 1 μl of 250 mM EDTA. Two microliters of 0.8 mg/ml proteinase K were added and mixtures were incubated at 45°C for 30 min to digest the topoisomerase II. Samples were mixed with 3 μl of loading buffer [60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanol FF, and 10 mM Tris (pH 7.9)] and subjected to electrophoresis in 1% agarose (MCB) gels in 40 mM Tris–acetate (pH 8.3), 2 mM EDTA at 5 V/cm for ~3 h. DNA bands were visualized by transillumination with ultraviolet light (300 nm), photographed, and quantified by scanning densitometry as previously described (Osheroff and Zechiedrich, 1987).

In order to map sites of topoisomerase II-mediated cleavage, reactions contained 120 nM topoisomerase II and 7 nM negatively supercoiled pBR322 plasmid DNA or 240 nM enzyme and 7 nM relaxed pBR322 DNA in a total of 50 μl of cleavage buffer that contained 5 mM $CaCl_2$. Samples were incubated at 30°C for 6 min. DNA cleavage products were trapped by the addition of 2.5 μl of 250 mM EDTA and 5 μl of 10% SDS (Osheroff and Zechiedrich, 1987). Five microliters of 0.8 mg/ml proteinase K were added and mixtures were incubated at 45°C for 30 min to digest the topoisomerase II. Samples were extracted with phenol, precipitated with ethanol, and digested with restriction endonuclease *Eco*RV (New England Biolabs) for 1 h at 37°C. Following digestion, mixtures were dephosphorylated with 3 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) at 37°C for 1 h, extracted with phenol, precipitated with ethanol, and labeled with 5 U of polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (6000 Ci/mmol, ICN). Reaction products were filtered through a column of Sephadex G-50 superfine, mixed with loading buffer and subjected to electrophoresis in 1.5% agarose gels as described above. Gels were dried and sites of topoisomerase II-mediated DNA cleavage were visualized by autoradiography using Kodak XAR film and a DuPont Lightning Plus screen.

Preparation of DNA substrates for electron microscopy

Covalently closed circular DNA topoisomers containing 0–5 negative superhelical twists (as determined by agarose gel electrophoresis or electron microscopy) were generated by incubating 80 nM pBR322 DNA with 10 U of calf thymus topoisomerase I in the presence of ~5 μg/ml ethidium bromide in 50 μl of cleavage buffer containing 5 mM $MgCl_2$ for 1 h at 37°C (Singleton and Wells, 1982). Linear DNA fragments 376, 753, 1540, and 1693 bp in length were generated by cleaving plasmid pBR322 with a mixture of restriction endonucleases *Eco*RI, *Bam*HI, *Pvu*II, and *Pst*I (New England Biolabs). All DNA samples were extracted with phenol, precipitated with ethanol, and redissolved in 5 mM Tris (pH 7.4), 0.5 mM EDTA prior to use.

Electron microscopy

Drosophila or calf thymus topoisomerase II (30 nM) was incubated with pBR322 DNA (10 nM circular or 7 nM linear fragments) for 6 min at 30°C in 20 μl of cleavage buffer which contained 5 mM $MgCl_2$, 5 mM $CaCl_2$, or no divalent cation. In some experiments, 10 mM HEPES (pH 7.9) was substituted for Tris in the cleavage buffer. Calf thymus topoisomerase I (30 nM) or *E.coli* ω protein (65 nM) was incubated as described above with 10 nM pBR322 DNA in cleavage buffer which contained no divalent cation. All topoisomerase–DNA complexes were fixed by the addition of 0.8% (final concentration) glutaraldehyde at 30°C for 6 min. Excess enzyme and buffer components were separated from complexes by filtration through columns of Sepharose 4B which were equilibrated in 5 mM HEPES (pH 7.4), 0.5 mM EDTA (Register *et al.*, 1987). Fractions containing

topoisomerase II–bound DNA (as determined by scintillation counting of reaction mixtures which employed tritiated pBR322 DNA) were applied to glow-discharged carbon-coated grids. Samples were dehydrated by sequential washes in water, 25%, 50%, 75% and 100% ethanol, and rotary shadowed at a 6° angle with tungsten (Griffith and Christiansen, 1978). In addition to the above, two sets of control protocols were followed. In some experiments, samples were applied directly to grids without gel filtration. In others, samples were filtered and applied to grids, but were not dehydrated prior to analysis. In all cases, samples were analyzed with a Hitachi H-800 electron microscope operated at 75–100 kV at ×10 000 to ×20 000.

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