On the Simultaneous Binding of Eukaryotic DNA Topoisomerase II to a Pair of Double-Stranded DNA Helices*

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Stabilization of crossings of pairs of DNA helices by binding of eukaryotic DNA topoisomerase II was studied by two types of experiments. In one, mixtures of yeast DNA topoisomerase II and supercoiled DNA were incubated with vaccinia virus topoisomerase, and the linking numbers of the DNA products were measured to quantitate supercoils that were constrained by the stoichiometrically bound yeast enzyme molecules. In parallel, the same yeast enzyme–supercoiled DNA mixtures were incubated with a nonhydrolyzable ATP analog AMPPNP (adenosine 5′- (β,γ-imido)triphosphate) instead of the vaccinia enzyme, and DNA linking number changes following the addition of AMPPNP were measured to monitor DNA transport mediated by the yeast enzyme and AMPPNP. In the second type of experiments, formation of knotted DNA rings by the addition of AMPPNP to mixtures of yeast DNA topoisomerase II and different topological forms of DNA rings was studied. These experiments indicate that binding of yeast DNA topoisomerase II to DNA crossings is significant, especially in low salt media containing Mg(II), and that this mode of binding strongly affects DNA knotting. It appears, however, that stabilization of DNA crossovers by the eukaryotic type II enzyme is not directly related to its DNA transport activity.

The elucidation of the modes of interaction between DNA and eukaryotic DNA topoisomerase II, a member of the type II DNA topoisomerase:DNA-dependent ATPase family, is of key importance in understanding the mechanism and biological roles of this enzyme. There is strong evidence that the binding of the enzyme to DNA protects a 25-base pair region against digestion by DNase I, and that near the center of this segment the enzyme makes a pair of transient scissions, one in each DNA strand, to open a DNA gate for the enzyme–DNA interaction. Another way of illustrating this is that an enzyme molecule bound to a DNA crossover would not be able to distinguish its substrate before and after strand passage of another double-stranded DNA segment (Lee et al., 1989) (for reviews on eukaryotic DNA topoisomerase II and other type II DNA topoisomerases including bacterial DNA topoisomerase I and phage T4 DNA topoisomerase, see Minwell and Gellert, 1986; Hsieh, 1990; Osheroff et al., 1991, and Caron and Wang, 1993).

In addition to binding to the 25-base pair DNA segment containing the DNA gate, eukaryotic DNA topoisomerase II can apparently interact with at least one other stretch of DNA. It has been known for some time that eukaryotic DNA topoisomerase II binds more strongly to positively or negatively supercoiled DNA than to linear or relaxed circular DNA (Osheroff and Brutlag, 1983; Osheroff et al., 1983; Osheroff, 1986, 1987). This higher affinity for supercoiled DNA is consistent with a preferential interaction between an enzyme molecule and a pair of DNA helices at a crossover or node of a DNA. Examination of DNA-bound eukaryotic DNA topoisomerase II by electron microscopy showed that the majority of enzyme molecules bound to a supercoiled DNA were present at crossings, and that enzyme molecules were also observed at crossovers on linear DNA (Zechiedrich and Osheroff, 1990). Preferential binding to DNA crossings was also reported for phage T4 DNA topoisomerase (Kreuzer and Huang, 1983).

Because the transport of one double-stranded DNA segment through another by a type II DNA topoisomerase necessarily requires the juxtaposition of two DNA segments at some stage of the enzyme’s catalytic cycle, the pair of DNA helices at an enzyme-bound DNA crossing were often thought to consist of a DNA segment containing the DNA gate (the G-segment), and a DNA segment to be transported through the gate (the T-segment). However, there is actually a mechanistic dilemma if the enzyme-bound DNA crossovers represent pairs of G- and T-segments. In contrast to bacterial gyrase, eukaryotic DNA topoisomerase II binds equally well to crossovers in positively or negatively supercoiled DNA; thus, an enzyme molecule bound to a DNA crossover would not be able to distinguish its substrate before and after strand transport. If an enzyme molecule would always retain a pair of G- and T-segments, then in the presence of ATP it might be expected to futilely transport the T-segment back and forth through the G-segment. Another way of illustrating this mechanistic dilemma is to consider the consequence of adding a nonhydrolyzable analog of ATP, such as AMPPNP. Under conditions such that all or a large fraction of crossovers in a negatively or positively supercoiled DNA are occupied by enzyme molecules, the addition of AMPPNP might be expected to drive a single DNA transport event at each crossover and thus invert the negatively supercoiled DNA to a positively supercoiled one or vice versa. This type of conversion has never been observed.

Stabilization of DNA crossings by type II DNA topoisomerases has also been implicated from their knotting of DNA rings. At a high molar ratio of phage T4 DNA topoisomerase to supercoiled DNA, knotted DNA rings are formed in the absence of ATP (Liu et al., 1980; Wasserman and Cozzarelli, 1991). Detailed characterization of the knotted products in the absence of ATP shows that they were formed by an enzyme-mediated DNA transport event between two more or less randomly located DNA segments, trapping topologically some of the nodes in the supercoiled DNA (Wasserman and

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1 The abbreviations used are: AMPPNP, adenosine 5′-(β,γ-imido)triphosphate; kb, kilobase pair(s).
Cozzarelli, 1991). In a second type of experiment, Drosophila DNA topoisomerase II was found to knot DNA rings at a high molar enzyme to DNA ratio in the presence of ATP (Hsieh, 1983). In the latter reaction, the use of a supercoiled DNA substrate was not necessary. The common feature of both types of reactions is that more enzyme molecules than DNA rings are necessary for knot formation; the excess enzyme molecules are presumed to stabilize DNA crossings.

To clarify how the stabilization of DNA crossings, the knotting reaction, and the transport of DNA segments by eukaryotic DNA topoisomerase II are interrelated, we have studied the stabilization of DNA crossings by yeast DNA topoisomerase II from linking number measurements of DNA rings relaxed with an excess of vaccinia virus DNA topoisomerase in the presence and absence of stoichiometric amounts of yeast DNA topoisomerase II. The efficiency of DNA transport by the bound yeast enzyme following the addition of AMPPNP, and knot formation accompanying such a process, were also examined. In agreement with previous conclusions drawn from different experimental approaches, we find that the type II eukaryotic enzyme can stabilize DNA crossovers, especially in a low salt medium, and that this stabilization is closely related to DNA knot formation. Our results indicate, however, that stable binding of the enzyme to a DNA crossover is not directly related to its catalysis of DNA transport.

EXPERIMENTAL PROCEDURES

Materials—Purification of Saccharomyces cerevisiae DNA topoisomerase II from yeast cells overexpressing a cloned yeast TOP2 gene was carried out as described previously (Worland and Wang, 1989; Lindsley and Wang, 1993). Quantitation of catalytically active enzyme in the homogeneous protein was done by titrating the protein with known concentrations of negatively supercoiled DNA under processive reaction conditions. A Poisson distribution of the homodimeric protein on plasmid DNA was assumed, and the concentration of protein that relaxes 64% of the supercoiled plasmid was taken to correspond to one active enzyme per plasmid (Lindsley and Wang, 1993). Active enzyme in a typical preparation was about one-half of the total protein concentration measured by the Coomassie-plus (Fierce Chemical Co.) method, using bovine serum albumin as the standard; in the preparation used in most of the measurements reported here, over 90% of the protein appeared to be active. Plasmid DNA purification, controlled nicking of plasmid DNA by DNase I, and the preparation of positively supercoiled DNA were done as described previously (Roca and Wang, 1992).

Methods—Protein-mediated retention of DNA to glass-fiber filter and two-dimensional agarose gel electrophoresis was carried out as described by Roca and Wang (1992) and Wang et al. (1983), respectively; minor modifications of the published procedures are specified in the relevant figure legends. Blot hybridization of DNA following gel electrophoresis and quantitation of the various forms of DNA in the gel blot by the use of a phosphor-imager were done as described by Lindsley and Wang (1993).

RESULTS

Restraining of Supercoils by the Stoichiometric Binding of Eukaryotic DNA Topoisomerase II to DNA—Relaxation of supercoiled DNA by an excess of vaccinia virus topoisomerase in the presence or absence of yeast DNA topoisomerase II was carried out to examine the restraining of DNA supercoils by the binding of the yeast enzyme in the absence of ATP. The results from a typical experiment are illustrated in Fig. 1A. Each of the four panels in Fig. 1A depicts the distribution of the various forms of a 3.1-kb plasmid following two-dimensional gel electrophoresis. The leftmost panel shows the pattern of a mixture of untreated negatively supercoiled DNA (arc labeled with a minus sign), and the same DNA after relaxation to completion by the vaccinia enzyme at 30 °C in a low salt buffer containing 50 mM Tris-HCl, pH 8, 50 mM KC1, 8 mM MgCl2, 7 mM 2-mercaptoethanol, and 100 μg/ml bovine serum albumin (spot labeled R). The arc of the negatively supercoiled DNA as well as the spot R represents a cluster of DNA topoisomers of different linking numbers, and individual topoisomers are often recognizable in autoradiograms of lighter exposures. The second panel from the left displays the electrophoretic pattern of the same negatively supercoiled DNA after incubation with approximately 18 yeast enzyme molecules/3.1-kb DNA ring. The distribution of DNA topoisomers in this sample is identical to that of the untreated sample in the leftmost panel, as catalysis of linking number change by intact type II eukaryotic enzyme is strictly dependent on the presence of ATP or its analog (Hsieh, 1990).

The topoisomer distribution of the DNA after incubation of the supercoiled DNA-yeast DNA topoisomerase II mixture with an excess of vaccinia DNA topoisomerase is, however, very different from that of the same DNA relaxed by the vaccinia enzyme in the absence of yeast DNA topoisomerase II (compare the broad arc of spots labeled R in the third panel from the left with the spot R in the leftmost panel in Fig. 1A). Quantitation of the two topoisomer distributions using a phosphor-imager shows that ∆Lk, the difference between the average linking number of the DNA relaxed in the presence of 18 yeast DNA topoisomerase II molecules/DNA and that of the same DNA relaxed in the absence of the yeast enzyme, was ~5.6.

Fig. 1. The resolution and detection of various forms of a 3.1-kb plasmid by two-dimensional agarose gel electrophoresis, blot-hybridization, and autoradiography. N, nicked rings; (-), negatively supercoiled plasmid; R, plasmid after relaxation by vaccinia virus topoisomerase; L, linear DNA; K, nicked knotted rings (discussed under "Results"). The sample shown in panel A1 contained a mixture of untreated negatively supercoiled DNA and the same DNA after treatment with an excess of vaccinia DNA topoisomerase at 30 °C in a medium containing 50 mM KCI plus buffer A (50 mM Tris-HCl, 8 mM MgCl2, 1 mM EDTA, 7 mM 2-mercaptoethanol, and 100 μg/ml bovine serum albumin). Samples shown in panels A2-A4 were obtained as follows: 0.1 pmol of plasmid was mixed with 1.8 pmol of yeast DNA topoisomerase II in buffer A plus 500 mM KCI, and the mixture was diluted with buffer A to give a final KCI concentration of 50 mM and split into three; the first was incubated at 30 °C for 10 min with no additions (panel A2), the second was incubated with vaccinia virus topoisomerase (0.1 μg/ml final concentration) at 30 °C for 10 min (panel A3), and the third was incubated with AMPPNP (2 mM final concentration) at 30 °C for 10 min (panel A4). Samples shown in panels B2-B4 were identically treated as their counterparts shown in panels A2-A4, except that the initial KCI concentration was 3 times higher to give a final KCI concentration of 150 instead of 50 mM. All reactions were terminated by the addition of sodium dodecyl sulfate and proteinase K to 1% and 100 μg/ml, respectively, and the reaction mixtures were incubated at 60 °C for 1 h prior to phenol extraction and ethanol precipitation of the DNA. Two-dimensional electrophoresis was carried out in 1% agarose gel in TBE buffer (100 mM Tris-borate, pH 8.3, 2 mM EDTA) plus 0.2 and 2 μg/ml of chloroquine diphosphate respectively in the first (top to bottom, 60 V, 16 h) and second dimension electrophoresis (left to right, 60 V, 8 h).
When the KCl concentration in the reaction buffer was increased from 50 to 150 mM, the results depicted in Fig. 1b were obtained. In this medium, $\Delta Lk$ of the 3.1-kb negatively supercoiled plasmid relaxed in the presence and absence of approximately 18 yeast DNA topoisomerase II/DNA was measured to be $-1.8$, much lower than the value $-8.6$ measured in the low salt buffer. These results and those from similar measurements at different molar ratios of yeast DNA topoisomerase II to DNA are tabulated in Table I.

Several control experiments suggest that the residual $\Delta Lk$ values observed in the experiments described above are due to binding of yeast DNA topoisomerase II to the crossovers in the supercoiled DNAs, which restrained these crossovers and prevented their removal by the type I vaccinia virus DNA topoisomerase. First, in the 50 mM KCl low salt medium, when a second negatively supercoiled DNA was added after the incubation of the yeast enzyme with the first DNA but before the addition of the vaccinia virus topoisomerase, relaxation of the second DNA was always complete; thus, the observed $\Delta Lk$ was not due to incomplete removal of the unconstrained supercoils by the viral enzyme (there is no exchange between free and topoisomerase II-bound DNA in this medium; see Roca and Wang (1992)). Second, the sign of $\Delta Lk$ of the DNA after exhaustive treatment with vaccinia virus topoisomerase in the presence of yeast DNA topoisomerase II is always the same as that of the DNA before treatment (see data in Table I). This finding is consistent with the stabilization of the crossovers in the original DNAs by the bound yeast enzyme molecules. Furthermore, when singly nicked instead of negatively supercoiled plasmid DNA was incubated with the yeast type II enzyme, ligation of the nick in the absence of ATP by Escherichia coli DNA ligase, which uses NAD as the cofactor, yielded topoisomers with the same linking number distribution as that of the ligation product of the nicked DNA without any bound yeast DNA topoisomerase II (results not shown). Thus, the binding of yeast enzyme molecules to DNA devoid of supercoils does not appear to cause a substantial change in the overall twist or writhe of the DNA.

Mg(II) Dependence of Yeast DNA Topoisomerase II Binding to DNA Crossings—The stabilization of crossovers in supercoiled DNA by yeast DNA topoisomerase II is Mg(II)-dependent. When relaxation of negatively DNA by the vaccinia enzyme was carried out in the presence or absence of yeast DNA topoisomerase II in the 50 mM KCl medium without Mg(II), the linking number distributions of the topoisomers were not significantly different in the two cases (Fig. 2). Filter binding experiments in the 50 mM KCl medium with and without Mg(II) also showed that the yeast type II enzyme-mediated retention of DNA to glass-fiber filters was very low in the absence of Mg(II) (Fig. 3).

The Trapping of DNA Crossings and DNA Transport by Yeast DNA Topoisomerase II—To test whether the trapping of DNA supercoils by eukaryotic DNA topoisomerase II correlates with its transport of DNA, AMPPNP was added to various enzyme-DNA mixtures to effect DNA transport, and the number of DNA transport events in each case was estimated from the difference in the average linking numbers of the DNA before and after the addition of the ATP analog. The results of two typical experiments are displayed in the rightmost panels of Fig. 1 (A and B).

The same DNA-yeast DNA topoisomerase II mixture used in the experiments illustrated in the two middle panels of Fig. 1A was used in these experiments. After incubation of the mixture with about 18 type II DNA topoisomerase molecules/3.1-kb plasmid in the 50 mM KCl low salt buffer, AMPPNP was added to 2 mM to effect DNA transport. From a comparison of the topoisomer distributions before (Fig. 1A, second

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**TABLE I**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>[KCl] (mM)</th>
<th>$\Delta Lk$ upon relaxation with vaccinia virus topoisomerase</th>
<th>$\Delta Lk$ upon AMPPNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>-0.06</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>-0.06</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>+0.03</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>-0.06</td>
<td>-8.6</td>
</tr>
<tr>
<td>5</td>
<td>150</td>
<td>-0.06</td>
<td>-1.8</td>
</tr>
</tbody>
</table>

$\Delta Lk$ is the specific linking difference of the DNA substrate.

$E$/DNA is the molar ratio of active dimeric yeast DNA topoisomerase II to plasmid; a 3.1-kb plasmid SpT18 was used in all experiments except experiment 3, in which a 2-kb plasmid pH624 was used.

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**Fig. 2.** Mg(II) dependence of the retention of crossovers in a supercoiled DNA by stoichiometrically bound yeast DNA topoisomerase II. Panels 1, 3, and 5, untreated negatively supercoiled DNA; panel 2, the same DNA after incubation with excess vaccinia virus topoisomerase in 50 mM KCl plus buffer A without Mg(II); panel 4, the same negatively supercoiled DNA after exhaustive relaxation with vaccinia virus topoisomerase in the presence of 6 yeast DNA topoisomerase II molecules/plasmid, in 50 mM KCl plus buffer A; panel 6, same as panel 4, except that relaxation was carried out in 50 mM KCl plus buffer A without Mg(II). Two-dimensional electrophoresis was carried out as described in the legend to Fig. 1, except that no chloroquine was present in the first dimension electrophoresis.

**Fig. 3.** Yeast DNA topoisomerase II-mediated retention of DNA on glass-fiber filters. Panels A–C show the electrophoretic patterns of the filtrate (lane 1), 1 M salt wash (lane 2), and sodium dodecyl sulfate wash (lane 3) of 1 pmol of negatively supercoiled DNA (panel A) or a mixture of 1 pmol of DNA and 1 pmol of yeast DNA topoisomerase II (panels B and C). The DNA shown in panel A was relaxed in 50 mM KCl plus buffer A. Following incubation at 30 °C for 10 min, the solution was filtered through a glass-fiber filter, and the filter was washed successively with buffer A plus 1 M NaCl and buffer A plus 0.5% sodium dodecyl sulfate, to give the filtrate (lane 1), the 1 M salt wash (lane 2) and the detergent wash (lane 3); see Roca and Wang (1992) for detailed descriptions. The samples shown in lanes 1–3 in panels B and C were similarly obtained from the equimolar plasmid-yeast DNA topoisomerase II mixtures incubated at 30 °C for 10 min in 50 mM KCl plus buffer A (panel B) or 50 mM KCl plus buffer A without Mg(II) (panel C). N and S mark the positions of the nicked and negatively supercoiled DNA, respectively.
panel from the left) and after the addition of AMPPNP (Fig. 1A, rightmost panel), there is a noticeable shift in the topoisomer intensities following the addition of the ATP analog; quantitation of the shift by the use of a phosphor-imager shows that the average linking number of the negatively supercoiled plasmid had increased by 1.8, corresponding to 1.8/2 or 0.9 events of duplex DNA transport in each plasmid (Brown and Cozzarelli, 1979; Liu et al., 1980; Hsieh and Brutlag, 1980). When the same experiment was carried out in the buffer containing 150 mM KCl, the AMPPNP-driven linking number change is much more pronounced (Fig. 1B, rightmost panel); phosphor-imager measurements indicate an increase of $L_k$ of 6.9, corresponding to 3.5 DNA transport events/plasmid. The calculated number of DNA transport events following the addition of AMPPNP from this and other experiments are tabulated in the last column of Table I.

Results in Table I show clearly that the AMPPNP-driven DNA transport by yeast DNA topoisomerase II is much more efficient in the 150 mM KCl medium than the 50 mM KCl medium. To test whether the salt effect on the AMPPNP-promoted DNA transport is reversible, the experiment depicted in Fig. 4 was carried out. A 2.95-kb negatively supercoiled DNA (leftmost panel, Fig. 4) was mixed with about 6 yeast DNA topoisomerase II molecules/plasmid in the 50 mM KCl medium. Following the addition of AMPPNP, the reaction mixture was split into two; one was quenched and then subjected to two-dimensional gel electrophoresis (Fig. 4, second panel from the left), and the other was adjusted to contain 150 mM salt, incubated for 10 min, and then analyzed (Fig. 4, third panel from the left). For the samples depicted in the fourth and fifth panels of Fig. 4, the order of salt concentration change was reversed; the DNA-enzyme mixture was initially in the 150 mM KCl medium, AMPPNP was added, and the KCl concentration was then lowered to 50 mM. It is clear from this experiment that the AMPPNP-driven change in $L_k$ by the yeast enzyme is dependent on the salt concentration of the medium at the time of addition of the ATP analog; change in salt concentration subsequent to AMPPNP addition induces no further linking number adjustment.

Trapping of DNA Crossovers and the Knotting of Duplex DNA Rings by Eukaryotic DNA Topoisomerase II—In the experiment depicted in the rightmost panel of Fig. 1B, a family of spots denoted by $K$ are present diagonally in the gel autoradiogram. These represent knotted nicked DNA rings (for the identification of knotted DNA rings, see Hsieh (1983) and Wasserman and Cozzarelli (1991)). Knotting of a duplex DNA ring in this fashion can be viewed as the topological trapping of DNA crossovers or nodes by the transport of one duplex DNA segment through another, or, in rarer cases, by multiple DNA transport events. Because the DNA substrate in the particular experiment initially contained both negatively supercoiled and nicked forms, there was some uncertainty in regard to whether the nicked knots were derived from the unknotted nicked rings or from the negatively supercoiled DNA that subsequently became nicked. The results depicted in Fig. 5 show, however, that knotting of nicked, positively supercoiled, or negatively supercoiled DNA can occur with comparable efficiency under the experimental conditions employed.

In the experiment shown in Fig. 5, the DNA samples were nicked prior to analysis by agarose gel electrophoresis to facilitate the identification of knotted rings. Knotted rings of different complexities, and linking number topoisomers within each knot class, can also be discerned directly in the two-dimensional gel electrophoresis patterns. In Fig. 6, the 2.95-kb DNA shown in panel A initially contained about 6 yeast DNA topoisomerase II/plasmid, and treatment with AMPPNP was carried out in the 50 mM KCl medium. To the right of the arc of topoisomers of the unknotted DNA, there are four discernible arcs labeled 3–6 in the figure; the presence of arc 3 can be attributed to the formation of three-noded knots or trefoils of varying linking numbers, and the other arcs the formation of topoisomers of knots with higher degrees of complexity. Addition of AMPPNP to the same enzyme-DNA mixture in the 150 mM KCl medium resulted in the

![Fig. 4. Salt dependence of the AMPPNP-driven DNA transport by yeast DNA topoisomerase II. A negatively supercoiled 2.95-kb plasmid, the two-dimensional gel electrophoretic pattern of which is shown in panel 1, was mixed with yeast DNA topoisomerase II at a molar ratio of 1 plasmid to 6 dimers to create a mixture of yeast DNA topoisomerase II molecules/plasmid in buffer A plus 500 mM KCl. The mixture was split into two halves, and diluted with buffer A to 50 and 150 mM KCl final concentration, respectively. After 10 min at 30 °C, AMPPNP was added to each to 2 mM and incubation was continued for 10 min. Buffer A or buffer A plus 500 mM KCl was then added to decrease or increase the KCl concentration in the samples; after 10 more min, reactions were stopped and processed for two-dimensional gel electrophoresis as described in the legend to Fig. 1. Panel 2, sample in 50 mM KCl plus buffer A throughout; panel 3, KCl changed from 50 to 150 mM following incubation with AMPPNP; panel 4, sample in 150 mM KCl plus buffer A throughout; panel 5, KCl changed from 150 to 50 mM following incubation with AMPPNP.](https://example.com/fig4.png)

![Fig. 5. Knotting of the various forms of a 2.95-kb plasmid by yeast DNA topoisomerase II. The samples loaded in lanes 1–3 in each panel initially contained 0, 2, and 6 dimeric yeast DNA topoisomerase II molecules/plasmid in 500 mM KCl plus buffer A. Following dilution with buffer A to reduce KCl concentration to 50 mM and incubation at 30 °C for 10 min, AMPPNP was added to each to effect DNA transport. Reactions were terminated 20 min afterward, and each sample was phenol-extracted and ethanol-precipitated. The recovered DNA samples were nicked in the presence of excess of ethidium (Barzilai, 1973; Hsieh and Wang, 1975) and analyzed by agarose gel electrophoresis in TBE plus 0.5 μg/ml ethidium bromide. The starting DNA samples used in the experiments shown in panels A–D were, respectively, negatively supercoiled with a specific linking difference of −0.06, negatively supercoiled with a specific linking difference of −0.02, nicked, and positively supercoiled with a specific linking difference of +0.03. N, L, and K indicate nicked, linear, and knotted DNA, respectively; S indicates the position of supercoiled DNA due to incomplete nicking of the DNA during DNAse I treatment.](https://example.com/fig5.png)
formation of a prominent arc of trefoils but few knots of higher complexity (Fig. 6, panel B). Increasing the molar ratio of enzyme to plasmid to 12 in the 150 mM KC1 medium increased the AMPPNP-dependent formations of knots that are more complex than the trefoil (Fig. 6, panel D); interestingly, at this higher enzyme to plasmid ratio, there was little formation of knots of any kind in the 50 mM KC1 medium (Fig. 6, panel C).

DISCUSSION

As described above, the modes of interaction between DNA and yeast DNA topoisomerase II were probed by linking number measurements of pBDNA relaxed by vaccinia virus DNA topoisomerase in the absence and presence of stoichiometric amounts of yeast DNA topoisomerase II, and by knotting of DNA rings upon addition of AMPPNP to mixtures of DNA and yeast DNA topoisomerase II. The results from both sets of experiments are consistent with the earlier interpretation, based primarily on electron microscopic observations (Zechiedrich and Osheroff, 1990), that a single type I1 eukaryotic DNA topoisomerase molecule can bind simultaneously to a pair of DNA helices at a crossover or node of a double-stranded DNA.

The stabilization of DNA crossings by eukaryotic DNA topoisomerase II appears to be particularly significant in media of lower salt concentrations. In a medium containing 50 mM Tris-HCl, pH 8, 50 mM KC1, 8 mM MgCl2, and 18 yeast DNA topoisomerase II molecules/3.1-kb plasmid DNA, approximately 9 negative supercoils in each plasmid were retained after exhaustive treatment with vaccinia virus DNA topoisomerase, corresponding to the stabilization of 9 crossings/plasmid; increasing the KC1 concentration to 150 mM reduced the retainable negative supercoils to about 2.

Under our experimental conditions, residual negative supercoils due to the presence of yeast DNA topoisomerase II were detectable only in the presence of Mg(II). In the absence of Mg(II), interaction between DNA and yeast DNA topoisomerase II was weak, and little protein-DNA complex was detectable by its retention to a glass-fiber filter. Zechiedrich and Osheroff (1990) observed, however, the preferential binding of Drosophila or calf thymus DNA topoisomerase II to duplex DNA crossovers in the presence or absence of divalent ions, and Sander et al. (1987) also reported that the presence of a divalent ion is not essential for the formation of a nitrocellulose filter-retainable complex between DNA and Drosophila DNA topoisomerase II. The cause of the discrepancy in Mg(II) dependence is obscure; differences in the purity of the enzyme preparations and in the intrinsic properties of DNA topoisomerase II from different organisms are some of the possibilities.

Whereas stabilization of DNA crossovers by eukaryotic DNA topoisomerase II is sufficient for the retention of supercoils in the presence of excess vaccinia virus DNA topoisomerase, the AMPPNP-driven formation of knotted DNA by eukaryotic DNA topoisomerase II requires the enzyme to serve a dual role of stabilizing the crossovers and transporting one DNA segment through another. Although it is conceivable that knotting may result from the topological trapping of nodes in a supercoiled DNA by the AMPPNP-driven action of a single type II enzyme molecule, a number of studies suggest that knot formation is much enhanced in the presence of ligands that bridge pairs of DNA segments (Liu et al., 1976, 1980; Haieh, 1983; Wasserman and Cozzarelli, 1991; Annan et al., 1992). In the experiment shown in panel C of Fig. 6, in the presence of 12 type II topoisomerase molecules/2.95-kb DNA, many nodes were presumably stabilized in the low salt buffer but few AMPPNP-promoted DNA transport events were detectable. Where AMPPNP-promoted DNA transport events were capable of topologically trapping these nodes had occurred; thus, knot formation was minimal. A reduction of the number of enzymes reduced the number of stabilized nodes but increased the number of DNA transport events, and knot formation was evident (Fig. 6A). Increasing the KC1 concentration reduces the number of stabilized nodes while improving even further the efficiency of DNA passage, leading to the efficient formation of predominantly trefoils (Fig. 6B); increasing the number of enzyme molecules per DNA ring in the same 150 mM KC1 medium increases the number of stabilized nodes, and thus both trefoils and knots of higher complexity were formed (Fig. 6D).

The knot formation results also reveal that the yeast enzyme can bridge two DNA segments in a nicked as well as supercoiled DNA ring; the yields of various knots from nicked DNA rings were not grossly different from those from supercoiled DNAs under the same conditions. It might seem contradictory that the yeast enzyme can knot a nicked DNA ring, a process which requires the trapping of several nodes, but there is no difference in the average linking number of the DNA upon ligation of the nick in the presence or absence of bound yeast DNA topoisomerase II molecules. It is expected, however, that crossovers of opposite signs are nearly equally stabilized by the binding of a eukaryotic type II DNA topoisomerase. Whereas a net number of crossovers of one sign or another may be trapped in a particular DNA molecule by a DNA transport event to yield a knotted ring, the average linking number of the entire population of ligated DNA molecules is unaltered because of the equal probability of trapping crossovers of opposite signs. This interpretation is consistent with the results of DNA knotting by the Drosophila type II enzyme in the presence of ATP (Haieh, 1983); relaxation of the originally supercoiled DNA is rapid in the presence of ATP, and knotting is apparently unrelated to the trapping of the unconstrained supercoils in the original DNA. Significantly, the optimal salt concentration for DNA knotting by the Drosophila enzyme was found to be around 100 mM, rather than the 150 mM optimum for the catalysis of DNA transport by this enzyme; the lower optimal salt concentration for knotting is most likely a reflection of the dual role of the enzyme in DNA knotting discussed earlier.

A major focus of the present work is to deduce whether the binding of eukaryotic DNA topoisomerase II to duplex DNA crossovers is directly related to the enzyme's transport of one DNA segment through another. Data presented under "Results" do not support the view that the pair of DNA helices
present at an enzyme-stabilized DNA crossover correspond to the G- and T-segments. First, if an enzyme bound crossover represents a T-segment posed for transport by the enzyme, the number of DNA transport events upon the addition of AMPPNP would be expected to increase with increasing number of such enzyme-bound crossovers. It was found, however, that increasing the molar ratio of yeast topoisomerase to plasmid from 6 to 18 increased the number of constrained supercoils per plasmid from 3.3 to 8.6 in the 50 mM KCl buffer, but the AMPPNP-driven DNA transport events in the same buffer actually dropped from 2.7 to 0.9 per plasmid. Second, under ionic conditions such that the type II eukaryotic enzyme binds well to DNA crossovers, the efficiency of DNA transport upon addition of AMPPNP is poor. In the presence of 18 yeast DNA topoisomerase II molecules per negatively supercoiled DNA in the 50 mM KCl medium, for example, only 0.9 DNA transport events occurred upon addition of AMPPNP, whereas many more DNA crossovers were constrained by the enzymes. Conversely, under ionic conditions that favor AMPPNP-promoted DNA transport by the type II enzyme, trapping of DNA crossovers is rather inefficient; in the presence of 6 type II enzyme molecules per negatively supercoiled DNA in the 150 mM KCl medium, less than 1 DNA crossover was retained, yet more than 5 DNA transport events were seen upon the addition of AMPPNP.

Here the numerology is clear; DNA transport does not require the binding of the enzyme to a DNA crossover at the time of AMPPNP addition.

The inverse correlation between the efficiencies of trapping DNA crossings and transporting DNA segments suggests that stable binding of a type II eukaryotic topoisomerase to a DNA crossover, which can occur in the absence of ATP or its analog, hinders rather than facilitates its DNA transport activity. A likely interpretation of this inverse correlation is that the AMPPNP-driven linking number change involves the transport of mainly segments not present at the enzyme-bound crossover; the stable binding of type II DNA topoisomerase molecules to many of the DNA crossings in a plasmid constrains DNA segmental movements necessary for the passage of T-segments through enzyme-bound G-segments. In a recent model, the ATP-dependent capture of a T-segment by a G-segment bound enzyme has been postulated to be a key step in the DNA transport reaction (Roca and Wang, 1992).

It has also been reported that eukaryotic DNA topoisomerase II binds preferentially and cooperatively to a class of DNA sequence elements that have been implicated in nuclear matrix or metaphase scaffold binding (Adachi et al., 1989). When increasing amounts of eukaryotic DNA topoisomerase II were added to a mixture of DNA molecules with and without such sequence elements, the enzyme first titrates quantitatively DNA molecules containing these elements, to the extent of 1 bound enzyme/200 base pairs of the entire DNA molecule. This mode of DNA binding by the eukaryotic enzyme has been implicated in a structural role of the enzyme in chromosome organization (see references in the text).

Whether the stable binding of type II eukaryotic DNA topoisomerase molecules to DNA crossings is related to a structural role of the enzyme in the higher order organization of chromosome is unclear; the identification of protein domains responsible for this mode of DNA binding might serve as a prelude for genetic and biochemical tests of this possibility.

REFERENCES


