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Interactions of *Drosophila* DNA topoisomerase II with left-handed Z-DNA in supercoiled minicircles

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ABSTRACT

The native form of *Drosophila melanogaster* DNA topoisomerase II was purified from Schneider's S3 tissue culture cells and studied with two supercoiled minicircle preparations, *mini* and *mini-CG*, 354 bp and 370 bp in length, respectively. *Mini-CG* contains a $d(CG)_7$ insert which assumes a left-handed Z-DNA conformation in negative supercoiled topoisomers with a negative linking number difference $-\Delta Lk \geq 2$. The interactions of topoisomerase II with topoisomer families of *mini* and *mini-CG* were studied by band-shift gel electrophoresis in which the individual topoisomers and their discrete or aggregated protein complexes were resolved. A monocolonal anti-Z-DNA IgG antibody (23B6) bound and aggregated only *mini-CG*, thereby confirming the presence of Z-DNA. Topoisomerase II bound and relaxed *mini-CG* more readily than *mini*. In both cases, there was a preference for more highly negatively supercoiled topoisomers. The topoisomerase II inhibitor VM-26 induced the formation of stable covalent DNA-protein intermediates. In addition, the non-hydrolyzable GTP analogue GTPγS inhibited the binding and relaxation activities. Experiments to detect topoisomerase cleavage sites failed to elicit specific loci on either minicircle preparation. We conclude that *Drosophila* topoisomerase II is able to bind and process small minicircles with lengths as short as 360 bp and negative superhelix densities, $-\sigma$, which can exceed 0.1. Furthermore, the enzyme has a preferential affinity for topoisomers containing Z-DNA segments and relaxes these molecules, presumably by cleavage external to the inserts. Thus, a potentially functional relationship between topoisomerase II, an enzyme regulating the topological state of DNA-chromatin in vivo, and left-handed Z-DNA, a conformation stabilized by negative supercoiling, has been established.

INTRODUCTION

Although primary DNA sequence plays the key role in gene expression, local variations in secondary structure generate targets for regulatory factors and in some cases act directly as control elements. Conformational transitions in DNA are closely linked to the formation and modulation of topological units, for which DNA topoisomerases are primarily responsible. Eucaryotic DNA topoisomerase II is a homodimer that relaxes positively and negatively supercoiled DNA by concerted scission of both strands, stereochemical reorientation (passage) of the two DNA strands such that the linking number, *Lk*, is altered by units of 2, and religation to reconstitute the double helix.[1–3] Covalent enzyme-DNA intermediates are involved in the mechanism.[4] The enzyme can knot, unknot, catenate, and decatenate relaxed closed circular DNA.[5] Studies of chromatin structure and assembly suggest the involvement of topoisomerase II in chromosome condensation[6,7] and as a structural protein, perhaps localized at the bases of radial loop domains in chromatin.[8–10] Topoisomerase II of *Saccharomyces cerevisiae* is essential for effecting the separation of sister chromatids after DNA replication.[11] A role in recombination[12] has been ascribed to the enzyme as well but remains to be demonstrated conclusively.

The mechanism by which topoisomerase II recognizes DNA and targets its enzymatic action *in vivo* is presumably related to the affinity of the enzyme for various DNA sequences and structures. In other studies, we have determined that *Drosophila* DNA topoisomerase II has a higher affinity for linear Z-DNA than B-DNA.[13] In this paper we have characterized the interaction of the enzyme with supercoiled minicircles, one of which contains a $d(CG)_7$ sequence in the left-handed Z conformation. These molecules provide unique advantages in studies of protein-DNA interactions because of the large differences in the relative electrophoretic migration of successive topoisomers, the large fractional contribution to the total mass and topology made by small inserts capable of assuming alternative conformational states, and the large band shift effects resulting from binding of proteins.[14,15]

MATERIALS AND METHODS

Preparation of supercoiled minicircles *mini* and *mini-CG*

Plasmids pAN700 and pAN701 (a gift from A. Nordheim) are derivatives of pUC7 constructed by insertion of the *Pvu* II fragment of pUC18 at the polylinker *Hind* II site.[14] pAN701 additionally contains a $d(CG)_7$ insert at the *Bam*H I site of the pUC18. Minicircles were generated according to the protocol

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of Nordheim and Meese.[14] In short, the plasmids were digested with *Eco*R I restriction endonuclease (New England Biolabs), the product fragments were dephosphorylated with alkaline phosphatase (Boehringer), and separated by 1% agarose gel electrophoresis. The smaller fragments containing the pUC18 sequences were excised from the gel and electroeluted. After ethanol precipitation, phenol/chloroform extraction, and ethanol reprecipitation, the fragments were end labeled with ^{32}P-γ-ATP by T4 polynucleotide kinase (New England Biolabs), and ligated at 0°C by T4 DNA ligase (New England Biolabs) in the presence of 2.2 μM ethidium bromide and 1 mM ATP. [The DNA concentration was kept below 1 mg/ml to promote intramolecular ligation.] After ligation the DNA was precipitated in the presence of a 10-fold excess of carrier tRNA, redissolved and analyzed by gel electrophoresis. Under these conditions a suitable distribution of topoisomers with a linking number difference, ΔLk, ranging from −5 to −2 was obtained. In the following, topoisomer numbers refer to the value of ΔLk.

Monoclonal anti-Z-DNA antibody 23B6

A monoclonal antibody (mAb), 23B6, was produced by immunization against chemically brominated poly[d(G-C)] [16] and subsequent hybridoma fusion.[17] The antibody binds to both linear and supercoiled DNA in the left-handed Z-DNA conformation in either low or high salt and has no strong preference for base sequence, indicating that it recognizes primarily the disposition of the DNA backbone. The binding affinity is at least three orders of magnitude higher for linear Z-DNA than for linear B-DNA.[17]

Drosophila topoisomerase II

Topoisomerase II was purified from a nuclear extract of Schneider's S3 *Drosophila* tissue culture cells by a new procedure.[13] Nuclei were extracted with 350 mM $(NH_4)_2SO_4$, dialyzed against low salt buffer solution, and chromatographed on a Pharmacia FPLC MonoQ ion exchange column, followed by a FPLC Superose sizing column. The fraction used for these studies was of high specific activity and contained no degraded enzyme. The purity was greater than 95% as determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a specific activity of $>3\times10^6$ units/mg. One unit is defined as the amount of enzyme necessary to relax 0.3 μg supercoiled plasmid DNA in 30 min at 30°C, in a solution containing 10 mM Tris-HCl, pH 7.9, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, and 20 μg/ml BSA.

Binding/activity reactions

The standard binding/activity assay containined ~1 ng of ^{32}P end-labeled supercoiled minicircles (~2,500−5,000 cpm) in a total volume of 8 μl of 10 mM Na-Hepes, pH 7.5, 60 mM NaCl, 8 mM $MgCl_2$, 0.1 mM spermidine, 0.03 mM spermine, and 1 mM ATP, including 1 μl of either anti-Z-DNA mAb or DNA topoisomerase II. The minicircle was introduced to the reaction mixture at room temperature for 5 min before addition of the protein. The complete mixture was incubated 30 min at 22°C, then 2 μl of 50% glycerol was added and the sample was loaded onto a 4−6% polyacrylamide gel (acrylamide: bisacrylamide ratio 30:1) in 0.5×TBE buffer (45 mM Tris-borate, pH 8.0, 0.1 mM EDTA) or a 1.5% agarose gel in 1×TBE buffer. For SDS (0.1%) gels, the reactions were stopped by the addition of 1 μl of 10% SDS. Where indicated, 1 μl of 5 mg/ml Proteinase K (Boehringer) was also added and the samples incubated overnight at 30°C prior to electrophoresis. The non-denaturing polyacrylamide and agarose gels were run at 4°C and the SDS containing gels at room temperature. After electrophoresis, the gels were dried and autoradiographed. The transilluminated films were digitized with a Photometrics Series 200 CCD camera. The images were processed on a Macintosh II/fx with TCL-Image (Delft Center for Image Processing) and NIH Image and rasterized for reproduction on a Linotype printer. The figures and scans represent the transmission of the films linearly. The assigment of topoisomers was taken from Nordheim and Meese;[14] it should be noted that all linear forms migrate at least six times faster than the supercoiled species and are not visualized on the polyacrylamide gels. The topoisomerase II inhibitor VM-26 was the gift of Bristol-Meyer; GTPγS, a non-hydrolyzable phosphorothioate analogue of GTP, was a gift of F. Eckstein.

RESULTS

The preferences of *Drosophila* DNA topoisomerase II for different closed circular and supercoiled DNA structures were tested with the minicircle gel retardation approach developed by Nordheim and Meese.[14] We prepared ^{32}P-labeled supercoiled minicircles, *mini* (354 bp) and *mini-CG* (370 bp) derived from pAN700 and pAN701, respectively. A diagram of the relative

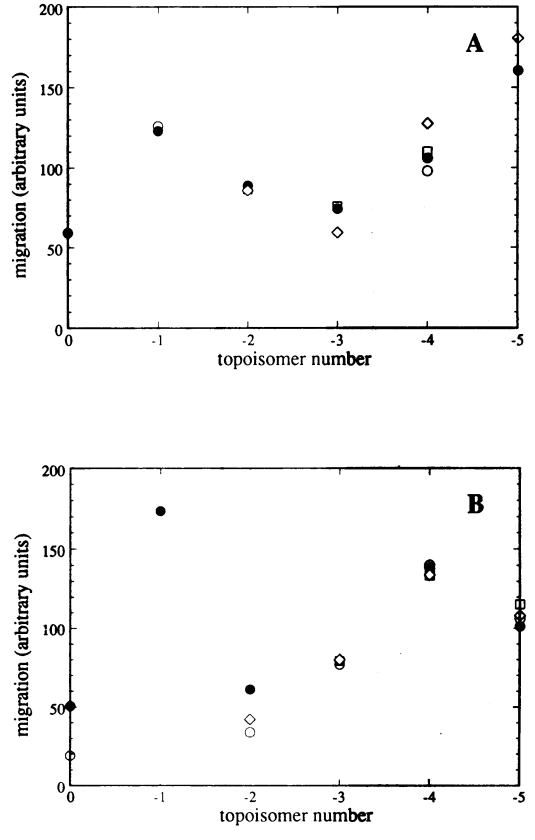

Figure 1. Relative electrophoretic mobility of minicircle topoisomers in non-denaturing PAGE. Data for the migration of (**A**) *mini* or (**B**) *mini-CG* in 4% polyacrylamide gels are derived from Nordheim and Meese [14] (●), or this paper, Fig. 2 (□), Fig. 3 (○), Fig. 5 (◇). The relative scales were adjusted to achieve the best coincidence between the patterns.

gel mobilities of *mini* and *mini-CG* topoisomer populations is depicted in Fig. 1 together with the reference data of Nordheim and Meese[14] used for making the assignments. *Mini-CG* contains a $d(CG)_7$ repeat that undergoes a transition to Z-DNA when *DLk* changes from −1 to −2, as evidenced by the greatly reduced mobility of the −2 and higher number topoisomers as well as retardation upon binding anti-Z-DNA mAb.[14,15] The *mini* sequence also contains an inverted repeat sequence which is probably extruded in topoisomers with $-\Delta Lk \geq 2$.[14,15] In *mini-CG*, cruciformation is inhibited by the more facile B−Z transition[18] until at least $-\Delta Lk > 4$.

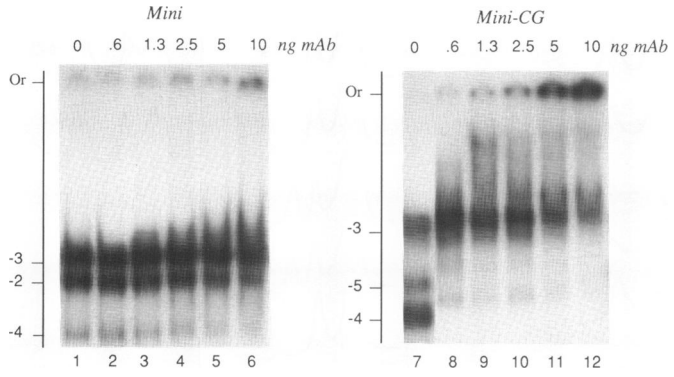

Figure 2. Binding of anti-Z-DNA mAb 23B6 to *mini* and *mini-CG* minicircles. One ng of ^{32}P-labeled minicircles was incubated with the indicated amounts of anti-Z-DNA 23B6 mAb and subjected to non-denaturing PAGE. *Mini*: lanes 1−6; *Mini-CG*: lanes 7−12. The ΔLk of the topoisomers are indicated at the left of each panel. Or: origin.

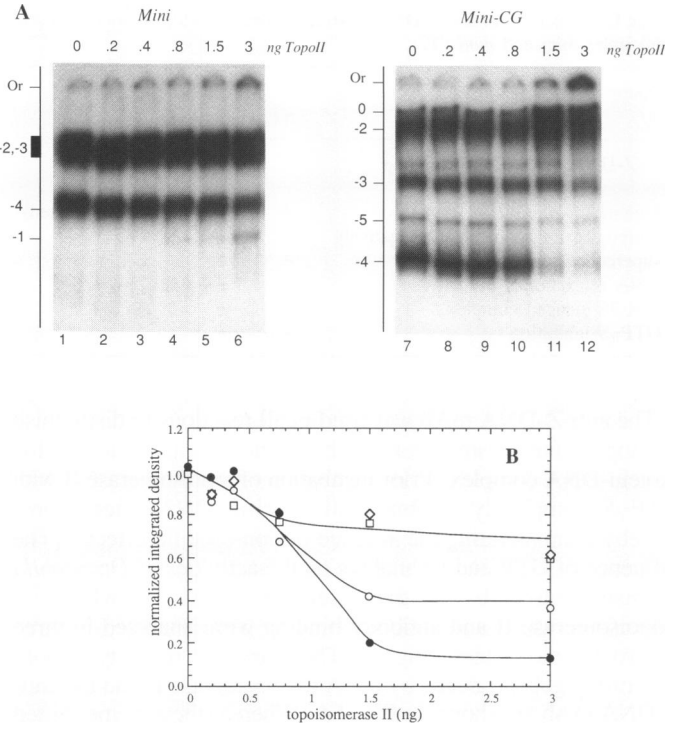

Figure 3. Binding of *Drosophila* topoisomerase II to *mini* and *mini-CG* minicircles. (**A**) One ng of ^{32}P-labeled minicircles was incubated with the indicated amounts of DNA topoisomerase II and subjected to non-denaturing PAGE. *Mini*: lanes 1−6; *Mini-CG*: lanes 7−12. Nomenclature as in Fig. 2. (**B**) Depletion of *mini* topoisomer −4 (□) and *mini-CG* topoisomers −4 (●), −5 (○), and −3 (◇) by topoisomerase. Data derived from panel **A** as described in the text. The integrated densities for each band were normalized by the value extrapolated from the first 4 points (lanes 1−4 and 7−10 in panel **A**).

Binding of anti-Z-DNA monoclonal antibody to minicircles

The effect of increasing concentrations of the anti-Z-DNA mAb on the mobility of different topoisomers is shown in Fig. 2. As in the case of other anti-Z-DNA antibodies,[14,15] the mAb 23B6 showed interactions only with the supercoiled *mini-CG* containing Z-DNA. The *mini* lacking a potential Z-DNA sequence did not interact with the antibody at these concentrations. From experiments with more diluted antibody, we determined that the affinity of the mAb is at least 500-fold higher for Z-DNA-containing *mini-CG* than for *mini*. No binding could be detected to any relaxed or linear forms of either minicircle preparation.

Binding of active *Drosophila* topoisomerase II to minicircles

After binding to supercoiled DNA, topoisomerase II can catalyze a topological relaxation. Thus, one expects a complicated pattern of interactions between the enzyme and its DNA substrate. Figure 3A shows the preferential disappearance of the −4 and −5 topoisomers of *mini-CG* concomitant with a parallel increase in slower migrating bands at the level of the −2 and 0 topoisomers, as well as the formation of insoluble complexes at the origin of electrophoresis. In contrast, *mini* showed a much reduced binding evidenced by a decrease of the −4 topoisomer only at the highest concentrations of topisomerase II and concomitant formation of very small amounts of the −1 topoisomer. Small amounts of insoluble protein-DNA complex were also detected. The titration data were analyzed further by logarithmically converting the digitized images to optical densities and integrating individual bands (Fig. 3B). A comparison of topoisomers −4 (*mini*) and −3, −4 and −5 (*mini-CG*) showed a similar relative decrease to ~80% upon adding topoisomerase II to a level of 0.75 ng. Addition of topoisomerase II to 3 ng reduced *mini* topoisomer −4 slightly more, i.e., to ~60%.

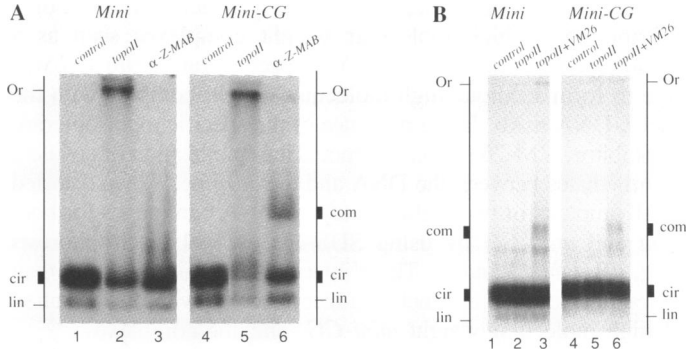

Figure 4. Analysis of anti-Z-DNA mAb and topoisomerase II complexes of *mini* and *mini-CG* minicircles by non-denaturating (**A**), and denaturing (**B**), agarose gel electrophoresis. (**A**) One ng of ^{32}P-labeled minicircles (lanes 1,4) was incubated with 3 ng DNA topoisomerase II (lanes 2,5) or 5 ng anti-Z-DNA mAb (lanes 3,6) and subjected to non-denaturing agarose gel electrophoresis. *Mini*: lanes 1−3; *Mini-CG*: lanes 4−6. The circular relaxed and supercoiled (cir) and linearized (lin) minicircles are indicated on the left of each panel. Or: origin. (**B**) One ng of ^{32}P-labeled minicircles (lanes 1,4) was incubated with 1.5 ng DNA topoisomerase II in the absence (lanes 2,5) or presence (lanes 3,6) of 30 μM VM-26 and subjected to SDS-agarose gel electrophoresis. *Mini*: lanes 1−3; *Mini-CG*: lanes 4−6. Nomenclature as in panel (**A**).

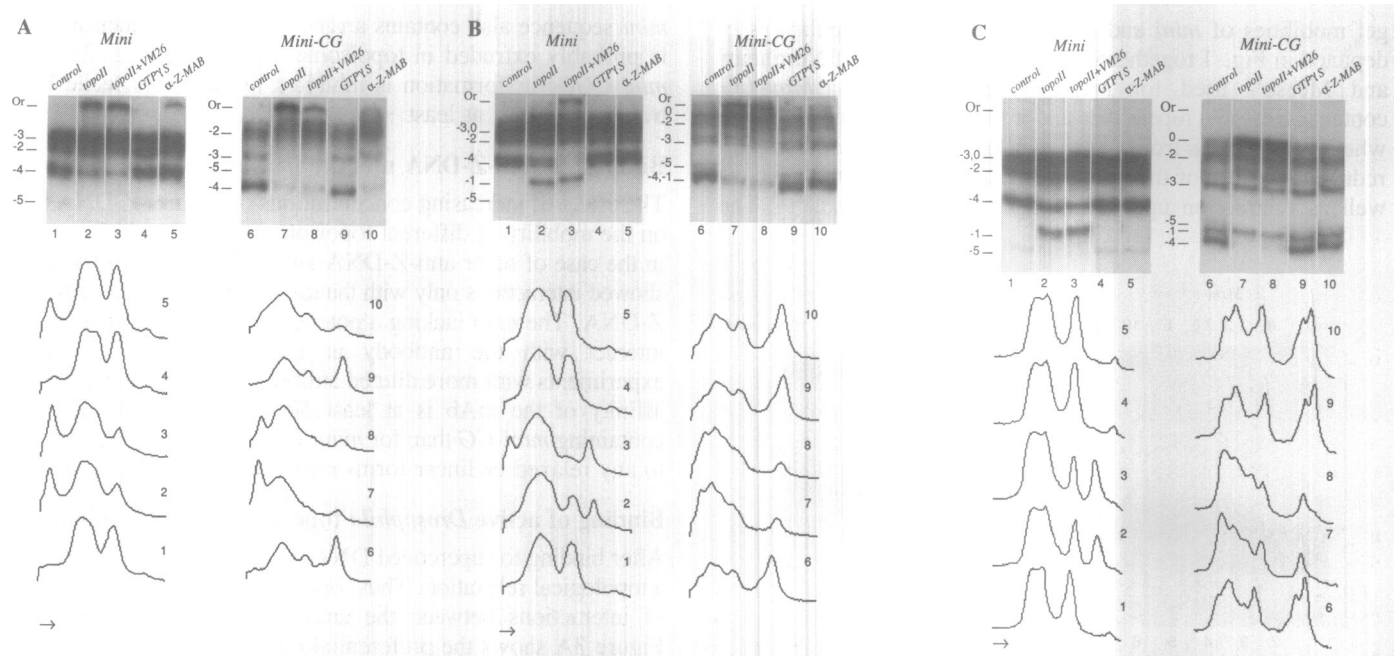

Figure 5. Analysis of topoisomerase II reactions in the presence of inhibitors and of anti-Z-DNA mAb complexes with *mini* and *mini-CG* minicircles. One ng of ^{32}P-labeled *mini* (lanes 1–5) or *mini-CG* (lanes 6–10) minicircles was incubated with 1.5 ng DNA topoisomerase II (lanes 2–4,7–9) in the absence (lanes 2,7) or the presence of 30 μM VM-26 (lanes 3,8) or 1 mM GTPγS (lanes 4,9), or with 10 ng (lane 5) or 1 ng (lane 10) anti-Z-DNA mAb and subjected to PAGE under non-denaturing conditions (**A**), or under denaturing conditions with SDS (**B**), and SDS after Proteinase K digestion (C). *Mini*: lanes 1–5; *Mini-CG*: lanes 6–10. The scanned profiles of each lane are displayed under the panels. Arrows indicate the direction of DNA migration. Other nomenclature as in Fig. 2.

However, *mini-CG* topoisomers −4, −5, and −3 were depleted more significantly (to ~ 10%, 40%, and 60%, respectively) with 3 ng topoisomerase II. This level of enzyme corresponded to ~0.5 topoisomerase II homodimers per minicircle.

Inasmuch as multi-enzyme complexes migrate together with insoluble aggregates in the polyacrylamide gels, we analyzed the binding reaction products by electrophoresis in more porous 1.5% agarose gels. Both *mini* and *mini-CG* were able to form insoluble aggregates with DNA topoisomerase II (Fig. 4A). However, *mini-CG* produced a more dramatic decrease in the supercoiled forms of the plasmid (lane 5) with a parallel formation of a distribution of high molecular weight complexes seen as a smearing of the radioactivity. As expected, only *mini-CG* was able to form a unique high molecular weight complex with the anti-Z-DNA mAb. In the presence of the specific topoisomerase II inhibitor, VM-26, which promotes the stabilization of covalent intermediates between the DNA and the enzyme,[4,19] we detected small amounts of two distinct enzyme-DNA complexes for both *mini* and *mini-CG* by using SDS-agarose gel electrophoresis (Fig. 4B, lanes 3 and 6). These intermediates migrate faster than the complexes seen in panel A, a fact in accord with the formation of high molecular weight *mini-CG* -enzyme complexes.

Further analysis of topoisomerase II binding and activity

Under our incubation conditions, both binding and enzymatic relaxation of the supercoiled DNA can take place simultaneously. We analyzed the reaction products by polyacrylamide gel electrophoresis run under conditions designed to distinguish between both the binding complexes and the different topoisomers (Fig. 5). The concentration of minicircles was about 4 times that of the enzyme in these reactions. The autoradiographs were digitized and evaluated quantitatively.

Table I. Interactions of anti-Z DNA mAb and *Drosophila* topoisomerase II with minicircles *mini* and *mini-CG*

Property	Minicircle	
	mini	*mini-CG*
Anti-Z-DNA mAb (23B6) binding	−	+
Topoisomerase II activities		
binding	+	++ (Z-DNA preferred)
preference for more highly negatively supercoiled topoisomers	+	+
DNA relaxation	+	++
VM-26 induced complex	+	+
GTPγS inhibition	+	+

The anti-Z-DNA mAb was used in all reactions to distinguish the topoisomer distribution of the *mini-CG* and to locate the protein-DNA complex. Prior incubation of topoisomerase II with GTPγS completely inhibited all specific DNA interactions, thereby demonstrating the absence of non-specific effects. [The influence of GTP and its analogs on the activities of *Drosophila* topoisomerase II are discussed in detail elsewhere.[13]] Topoisomerase II and antibody binding were analyzed in three electrophoretic systems (Fig. 5). The changes in mobility on non-denaturing gels produced by DNA topoisomerase II and the anti-Z-DNA mAb are shown in Fig. 5A. Whereas the enzyme shifted the −5, −4, and −3 topoisomers of *mini-CG* (lane 7) to higher positions corresponding to slower migration, it complexed only the −5 and partially the −4 topoisomers of *mini* (lane 2). Both minicircles formed insoluble aggregates at the origin. In contrast, almost no detectable complex formed with 10-fold higher concentrations of anti-Z-DNA mAb incubated with *mini* (lane 5) relative to that used to retard the entire ensemble of supercoiled topoisomers of *mini-CG* (lane 10).

The same reaction samples as seen in Fig. 5A were analyzed by electrophoresis in the presence of the protein denaturant SDS (Fig. 5B). SDS dispersed most of the insoluble aggregates and led to the release of the some topoisomers from the enzyme-DNA complexes. Comparing lanes 2 (*mini*) in panels A and B, it is evident that the enzyme-DNA complex contained both −4 and −1 topoisomers. Since the action of topoisomerase changes the DNA linking number in steps of 2, we interpret these results as changes in *DLk* from −4 to −2 and from −3 to −1. Addition of VM-26 to the reaction (lanes 3, 8) resulted in very small amounts of adduct originating predominantly from the −4 topoisomer (see also panel C).

In the case of *mini-CG* almost all of the topoisomers were complexed and SDS treatment released only about 60% (mostly as the relaxed 0 topoisomer), indicating the existence of a more stable enzyme-DNA complex than that formed with the *mini*. The most negatively supercoiled topoisomers (−5, −4) reacted preferentially and were relaxed most completely. The overlapping of topoisomers −1 and −4 under the particular running conditions for gel electrophoresis did not allow a determination of the fate of the −3 topoisomer. No increase in the amount of enzyme-DNA complex was seen in the SDS gel in the reactions containing VM-26 (lanes 3, 8).

In view of the stability of topoisomerase-DNA complexes with *mini-CG*, we used digestion with Proteinase K in addition to SDS to analyze the actual topological changes in the DNA brought about by the enzyme reaction (panel C). All the insoluble aggregates disappeared and it was possible to distinguish between the topoisomers −1 and −4 of *mini-CG* as well as follow the transition from −3 to −1. All of topoisomers −5 and −4 of *mini-CG* underwent relaxation as well as some of the −3 as indicated by the appearance of a new band at the −1 mobility (lane 7). The appearance of −1 topoisomer in *mini* indicated relaxation of −3 (lane 2) whereas more than half of the −4 remained after release from the enzyme complexes (lane 2).

The *mini* and *mini-CG* minicircles do not contain the *Drosophila* DNA topoisomerase II consensus cleavage sequence defined by Lee et al.[20] In addition, we note that cleavage is very inefficient on these substrates compared with the activity of the same enzyme fraction on supercoiled plasmid DNA. Nonetheless, we tried to map specific double- and single-strand DNA breaks in the absence or the presence of VM-26 by a method similar to Udvardy et al.,[21] albeit without success. Thus, relaxation of the *mini* and *mini-CG* seems to have occurred by scission at a number of alternative sites.

DISCUSSION

This study demonstrates that *Drosophila* topoisomerase II is able to bind and act on small minicircles with lengths of ~360 bp and negative superhelix densities, $-\sigma$, exceeding 0.1. Table I summarizes the differences in interaction of *Drosophila* topoisomerase II with the small supercoiled DNAs. There is a significantly higher affinity of the enzyme for the *mini-CG* minicircle containing Z-DNA than the control *mini* construct lacking the $d(CG)_7$ insert. Both minicircle preparations share ~300 potential (overlapping) non-specific B-DNA binding sites for topoisomerase II and we surmise that these dominate the initial phase of the titration shown in Fig. 3. However, at higher topoisomerase II concentrations an additional binding capacity is expressed in *mini-CG* topoisomers, particularly −4 and −5, to an extent which correlates with migration velocity, and thus writhe. That is, the topoisomers expressing the Z conformation in the $d(CG)_7$ insert and exhibiting the greatest topological stress (writhe) bind topoisomerase II best. Another feature of this model is exclusive binding in that the small minicircles can probably accomodate one, or at most two, topoisomerase II molecules. One can estimate that its affinity for the left-handed region relative to that for B-DNA sites is substantially higher. The Z-DNA insert accounts for only 3.5% of the DNA of the *mini-CG* and may therefore constitute a single binding site. If so, the affinity of *Drosophila* topoisomerase II for left-handed Z-DNA compared to that for non-specific B-DNA sites is 1 or 2 orders of magnitude higher, an observation consistent with our data on the enzyme binding to linear Z- and B-DNAs.[13]

We have observed that the enzyme forms stable, high molecular weight complexes with the *mini-CG* (Figs. 4, 5A & B). Topoisomerase II has been shown to assist in the condensation of chromatin in vitro[7] and to form large networks with oligo(dG).[22] In addition, it constitutes a major protein of interphase scaffolds [8−10] where it is postulated to bind at the base of DNA loops. All of these data suggest that the enzyme forms stable DNA-enzyme complexes which may or may not involve the active site for DNA scission and result in concomitant relaxation. The formation of higher order aggregates argues strongly for more than one of these binding sites per homodimer and/or strong protein-protein interactions in the DNA-protein complex.

The inverted repeat sequence contained in the *mini* circles can form a cruciform in negatively supercoiled topoisomers. A recent report by Howard et al.[23] demonstrates that *Drosophila* topoisomerase II binds preferentially to bends, kinks and bubbles in DNA. If the extruded sequence expresses any of these unusual structures (e.g. the terminal loops of the cruciform), the *mini* may not reflect the general mode of interaction of topoisomerase II with B-DNA. However, we note that the enzyme showed a higher affinity for and formed more stable complexes with *mini-CG* in which the cruciform is not extruded. Thus, the influence of the $d(CG)_7$ insert in the Z-conformation was predominant. The lack of specific breakage points in either case indicates that the binding and cutting sites are not necessarily synonymous. We propose, in fact, that the enzyme binds preferentially to special DNA conformations such as Z-DNA but that cutting occurs elsewhere. This could occur via action of a second site on the enzyme on contiguous regions of the DNA or more remote segments rendered accessible due to the very compact structure of the supercoiled minicircle, or by translation of the enzyme away from the primary (Z-DNA) binding site.

Our data lead to the hypothesis that *Drosophila* topoisomerase II *in vivo* has an inherent bias for DNA regions of high negative superhelicity, binding particularly to those with left-handed Z-DNA elements, and perhaps to other non-B-DNA conformations as well. Inasmuch as topoisomerase II acts enzymatically to alter DNA topology, these features suggest a potential feedback mechanism for the regulation of chromatin organization.

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