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Eukaryotic topoisomerase II cleavage of parallel stranded DNA tetraplexes

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ABSTRACT

A guanine-rich single-stranded DNA from the human immunoglobulin switch region was shown by Sen and Gilbert [Nature, (1988) 334, 364 – 366] to be able to self-associate to form a stable four-stranded parallel DNA structure. Topoisomerase II did not cleave the single-stranded DNA molecule. Surprisingly, the enzyme did cleave the same DNA sequence when it was annealed into the four-stranded structure. The two cleavage sites observed were the same as those found when this DNA molecule was paired with a complementary molecule to create a normal B-DNA duplex. These cleavages were shown to be protein-linked and reversible by the addition of salt, suggesting a normal topoisomerase II reaction mechanism. In addition, an eight-stranded DNA molecule created by the association of a complementary oligonucleotide with the four-stranded structure was also cleaved by topoisomerase II despite being resistant to restriction endonuclease digestion. These results suggest that a single strand of DNA may possess the sequence information to direct topoisomerase II to a binding site, but the site must be base paired in a proper manner to do so. This demonstration of the ability of a four-stranded DNA molecule to be a substrate for an enzyme further suggests that these DNA structures may be present in cells.

INTRODUCTION

Topoisomerases have been implicated in events such as transcription (1–3), DNA replication and segregation of DNA molecules prior to cell division (4–7). The high concentration of topoisomerase II in the nuclear matrix/chromosome scaffold additionally suggests that the protein is important in some aspects of chromosome architecture (8–11). In terms of the enzymatic activity, an important feature of these enzymes is their ability to discriminate between different DNA sequences (12) and DNA structures at which breakage/rejoining of DNA strands proceeds (13). DNA cleavage by topoisomerase II is blocked by DNA triplex formation (I.K. Chung, J.R. Spitzner and M.T. Muller, manuscript submitted). Topoisomerase II reacts vigorously at certain sites and ignores others; for example, we recently reported that topoisomerase II is especially reactive with alternating purine-pyrimidine repeat sequences because the consensus recognition site (12) is reiterated at every other base (14). These repeats are associated with gene conversion and recombination events (15,16) suggesting that topoisomerase II could additionally participate in these processes in the cell. Three observations support the idea. First, one of the best topoisomerase II cleavage sites identified to date corresponds to a hotspot for recombination in the β-globin gene (see (14) and references therein). Second, prokaryotic type II topoisomerases have been implicated in illegitimate recombination and deletions between direct repeats (17,18). Third, recombination events have been reported *in vitro* with calf thymus topoisomerase II (19).

Furthermore, Rose *et al.* suggested that topoisomerase II is required at the time of chromosome segregation in meiosis I for the resolution of recombined chromosomes (20). The mechanism by which topoisomerase II might act in strand exchange and transesterification is unknown. In this work, however, we report the surprising observation that although topoisomerase II does not cleave single stranded substrates, it does cleave the same DNA when annealed to form a novel structure characterized as parallel tetraplex DNA (21). These data support the notion that each protomer can react independently with a cleavage site on each DNA strand.

MATERIALS AND METHODS

Materials

Topoisomerase II was purified from chicken blood as described previously (22). Restriction enzymes were purchased from Bethesda Research Laboratories, T4 polynucleotide kinase from United States Biochemical Corp., and gamma-[^{32}P]-ATP from ICN. Oligonucleotides were synthesized on an Applied Biosystem DNA synthesizer from Biochemical Instrumentation Center at The Ohio State University. The topoisomerase II inhibitors *m*-AMSA (4′-(9-acridinylamino) methanesulfon-*m*-anisidide) was provided by the National Cancer Institute, division of synthetic products.

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Isolation of M4 and M4R4 DNA structures

M4 DNA was prepared as described by Sen and Gilbert (23). The M oligomer (2 μg) was labeled at its 5′ end using polynucleotide kinase and gamma-[^{32}P]-ATP; following ethanol-precipitation was resuspended in 10 μl of buffer A (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA). The reaction was incubated for 20 hrs at 50°C and the M4 and monomer M DNAs were purified from a 6% nondenaturing preparative polyacrylamide gel (21). M4R4 DNA was prepared by resuspending 1 μg of gel purified M4 DNA in Buffer B (10 mM Tris-Cl, pH 8.0, 750 mM KCl, 1 mM EDTA) and adding 4 μg of unlabeled oligo R (final volume of 15 μl) followed by incubation at room temperature for 1 hr. The MR and M4R4 species were cut out of a polyacrylamide gel, eluted into buffer B by diffusion, ethanol-precipitated and resuspended in Buffer B.

Topoisomerase II cleavage reactions on M4 and M4R4 DNA structures

Cleavage reactions were performed in a final volume of 20 μl in cleavage buffer (30 mM Tris-HCl, pH 7.6, 60 mM KCl, 8 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 3 mM ATP, 30 μg/ml BSA) and specified labeled DNA structures (usually 1,500 cpm or 1 ng). The topoisomerase II inhibitor, *m*-AMSA, was added as specified in each experiment and the reactions were initiated by addition of 4 units of purified chicken topoisomerase II, incubated 30 min at 30°C and terminated by addition of SDS to 1% followed by incubation with 50 μg/ml proteinase K at 56°C for 30 min. Samples were precipitated with ethanol and resuspended in sequencing gel loading buffer prior to loading onto 20% sequencing gels. Topoisomerase II cleavages on equal concentrations of gel purified M, M4, M4R4 and MR DNA were performed as described previously (12). All DNA forms were used directly (without storage) in topoisomerase cleavage assays. Stability of the M4 and M4R4 DNAs were evaluated concurrently by analyzing duplicate reactions in a 6% native polyacrylamide gel. Less than 1% of the M4 or M4R4 DNA were converted back to M or MR DNA, respectively, after incubation in topoisomerase II cleavage reactions (data not shown).

Religation of topoisomerase II cleavage intermediates

After incubation for 30 min at 30°C, topoisomerase II cleavage reactions were added EDTA (10 mM final) and NaCl (200 mM final concentration) to trap the covalent topoisomerase II-DNA cleavage complexes and then were incubated for 5 min at 30°C.

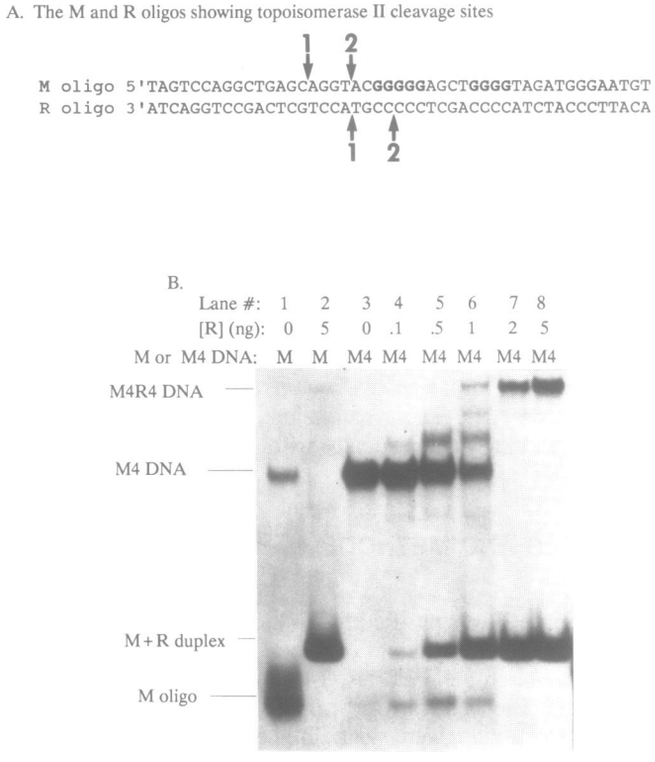

Figure 1. Analysis of electrophoretic mobilities of oligonucleotides derived from the switch region of immunoglobulin heavy chain (20,22). The sequence of oligonucleotides M and R is shown in A as duplex DNA, with the guanine repeats important in parallel strand pairing shown in bold face (20). Topoisomerase II sites are marked 1 and 2. Panel B shows a native polyacrylamide gel of end-labeled M oligo in various forms. M oligo, monostrand M; R oligo, monostrand R; MR duplex, Watson and Crick double stranded DNA; M4, four strands of M; M4R4, M4 complexed with four strands of complementary R. Lane 1, oligo M and the M4 complex; lane 2, MR duplex; lane 3, purified M4 complex; lanes 4–8, purified M4 complex incubated with indicated amounts of R oligonucleotide.

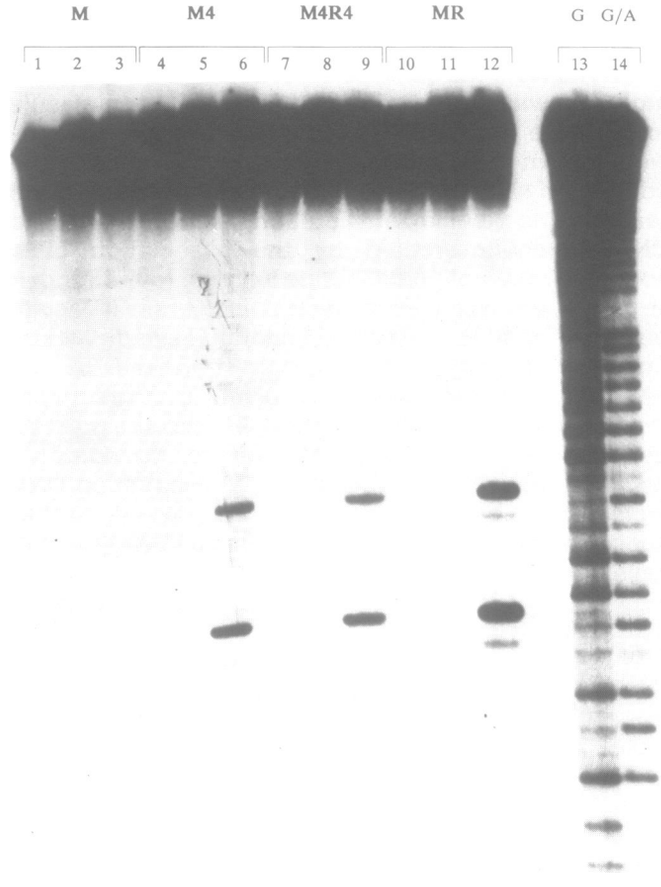

Figure 2. Topoisomerase II cleavages on tetraplex and duplex DNAs. Topoisomerase II cleavages were carried out with 5′ end labeled monostrand M DNA (lanes 1–3), tetraplex M4 DNA (lanes 4–6), octaplex M4R4 DNA (lanes 7–9) and duplex MR DNA (lanes 10–12). In each set of three samples, the first contained DNA and *m*-AMSA, the second and third contained DNA and 4 units of purified chicken topoisomerase II, and the third included 50 μg/ml *m*-AMSA. Chemical sequencing ladders are shown in lanes 13 and 14. Samples were analyzed on a 20% sequencing gel.

Religation was initiated by addition of $MgCl_2$ (5 mM final concentration) (24). After 10 min, reactions were terminated with an equal volume of 2% $NaDodSO_4$, and the DNA was phenol extracted and analyzed on a 20% polyacrylamide sequencing gel.

Recovery of topoisomerase II/DNA intermediates by $NaDodSO_4$/KCl precipitation

After 30 min at 30°C, topoisomerase II cleavage reactions (20 μl) were terminated by the addition of 0.1 vol of 10% SDS, followed by 0.25 ml of 10 mM Tris-HCl, pH 7.5, 20 μg BSA/ml, 20 μg calf thymus DNA/ml, and 1% SDS. The mixtures were vortexed and placed in a 37°C bath for 2−4 min followed by addition of 0.025 ml of 2.5 M KCl. After vortexing a second time, mixtures were placed on ice for 10 min. The precipitate was collected by centrifugation using a microfuge (2 min at 4°C) and was resuspended in 1.0 ml of cold TKE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM KCl) followed by washing twice. The final precipitate was resuspended in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), digested with proteinase K (50 μg/ml) to remove bound proteins, and ethanol-precipitated (22).

RESULTS

Formation of tetraplex M4 DNA

Sen and Gilbert (21,23) reported that an immunoglobulin sequence readily adopts a tetraplex parallel strand structure that is paired at the guanine tracts through Hoogsteen bonds. The single stranded 47 base oligonucleotide (oligo M, see Fig. 1A) self-anneals to form tetraplex DNA (referred to as M4, Fig. 1B, lane 1) which has a lower electrophoretic mobility relative to the monomer. M4 DNA can be isolated from a preparative gel and is very stable once formed under conditions specified by Sen and Gilbert (23); reloading a second gel confirms this observation (lane 3). From Fig. 1B (lanes 3−8) it can be seen that M4 DNA hybridized with complementary oligo R to form an 8-stranded structure (called M4R4) (21). Under the conditions used in these experiments (22), the M4R4 octaplex was quite stable even after incubation in topoisomerase II cleavage buffer, with less than 1% breakdown to duplex DNA (data not shown).

Topoisomerase II cleavages on MR duplex and tetraplex DNA

Topoisomerase II reactions were carried out on uniquely end labeled fragments and the products were sequenced (12,22). Two sites in the MR DNA duplex display significant homology to the topoisomerase II consensus sequence (12,25); these are labeled sites 1 and 2 (Fig. 1A). Topoisomerase II cleaved the MR duplex at the predicted sites (Fig. 2, lane 12), in a reaction containing the inhibitor *m*-AMSA. The M oligo, however, like other single stranded DNA molecules (data not shown), was not cleaved by topoisomerase II (Fig. 2, lanes 1−3). In contrast, when topoisomerase II was incubated with purified M4 tetraplex (or M4R4 octaplex) the enzyme cleaved the parallel strand structure at the same two sites as in the B-DNA (compare lanes 6 and 12). Although M4R4 DNA may convert to B form DNA, we do not know if M4R4 octaplex or MR duplex is being cleaved. What is significant is that M4 tetraplex has no complementary strand and must be cleaved by the enzyme since M monostrand is not cut.

Religation and $NaDodSO_4$/KCl precipitation of topoisomerase II cleavages on MR duplex and tetraplex DNA

It was necessary to determine whether the reaction intermediates trapped on tetraplex structures were identical to those with duplex DNA. The first test showed that, prior to protein denaturation, the cleavage reaction could be reversed by addition of EDTA and salt (Fig. 3, lanes 9,13,17) (24). This result confirms that topoisomerase II engages M4 DNA in a normal process of breaking and religating the sugar-phosphate backbone of DNA. The second test showed that the DNA cleavage was accompanied by formation of covalent protein-DNA intermediates that could be recovered by $NaDodSO_4$ and KCl precipitation (Fig. 3, lanes 23,26,29); this procedure allows selective recovery of stable

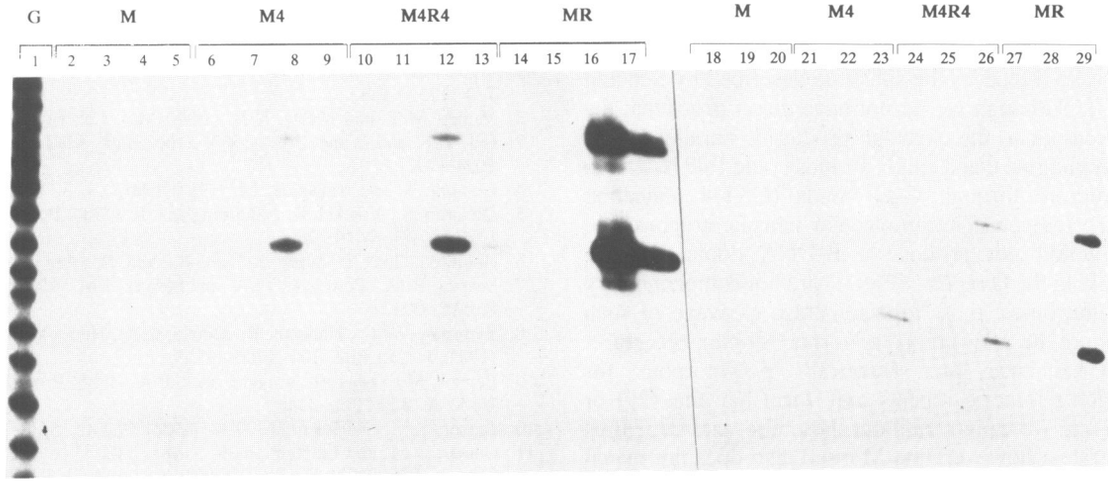

Figure 3. Topoisomerase II cleavage intermediates with DNA tetraplexes and duplexes. Topoisomerase II cleavages were carried out as in Fig. 2 legend on 5′ end labeled monostrand M (lanes 2−5), tetraplex M4 DNA (lanes 6−9), M4R4 DNA (lanes 10−13), and duplex MR DNA (lanes 14−17). For each set of four reactions, the lanes correspond to: DNA alone (first reaction); DNA and enzyme (second reaction); DNA, enzyme and *m*-AMSA (third reaction); DNA, enzyme, and *m*-AMSA followed by religation of cleavage intermediates as described in Materials and Methods (fourth reaction). Lane 1 is a chemical sequencing marker (G/A). In lanes 18−29, topoisomerase/DNA intermediates were recovered by $NaDodSO_4$/KCl precipitation as described in Materials and Methods (13): M DNA (lanes 18−20), M4 DNA (21−23), M4R4 DNA (lanes 24−26) and duplex DNA (lanes 27−29). For each set of three lanes, complexes were recovered from the following reactions: first, a control (no enzyme); second, enzyme alone; third, enzyme plus *m*-AMSA.

DNA protein complexes (26). The topoisomerase II reactivity of the M4R4 octaplex structure was identical to that of M4 and MR duplex. Additional experiments with M4R4, in which the R strand was labeled, revealed that topoisomerase II also cleaved this strand, at the sites indicated in Fig. 1A (data not shown). To determine whether other proteins were reactive towards the octaplex M4R4 structure, we performed similar experiments with restriction enzymes. Under conditions for topoisomerase II activity, the restriction enzymes RsaI (the site is exactly at topoisomerase II cleavage site number 2 in Fig. 1) and DdeI (this site is 5 bases 5′ of site number 1) cut the duplex MR efficiently; however, no restriction was detected with M4R4 (data not shown). It appears that topoisomerase II can bind, cleave, and religate M4 DNA in a dynamic process similar or identical to duplex DNA.

DISCUSSION

Previous observations indicated that topoisomerase II was enzymatically active only on duplex DNA molecules (or on single-stranded molecules, but only at sites which can adopt a double-stranded conformation, such as hairpin formation; see (27, 28), for example). It was, therefore, not unexpected that the 47 base M oligo monomer was not cleaved by topoisomerase II (Fig. 2, lanes 1−3 and Fig. 3, lanes 2−5), nor was it cross-linked to the protein (Fig. 3, lanes 18−20); in fact, even long-overexposed autoradiograms showed no cleavage of the M oligo (data not shown). Thus, we were quite surprised to find that when the M oligo was allowed to self-associate to form a tetraplex (M4) structure and was then purified and treated in a manner identical to that for the M oligo, the M4 molecule clearly was cleaved by topoisomerase II.

These results suggest that the M oligo contains the sequence information necessary to direct the action of the enzyme to its cleavage sites; however, topoisomerase II does not cleave single stranded DNA. We demonstrated that when a single stranded DNA is annealed with itself in the absence of a complementary sequence, a structure results that can be recognized and cleaved by topoisomerase II. Since we isolated a structure using conditions optimized for parallel stranded tetraplex DNA (23), we infer that the topoisomerase II cleaves the substrate described by Sen and Gilbert (21,23). Although we do not have direct proof that the immediate precursor to the cleavage product is parallel DNA, it is difficult to imagine that a single strands could fold back into a hairpin structure through G-G bonds (G5,G4 annealing intramolecularly). Even if intramolecular hairpin structures do form, these would not produce a B-DNA duplexes since sequences flanking the G repeats (Fig. 1) are not complementary repeats or palindromes; thus, topoisomerase cleavage of such molecules cannot be viewed as a normal duplex structure. Guanine rich sequences are structurally polymorphic; for example, G-rich telomeric repeats may form hairpins (29) or quartets (30), and we cannot rule out these alternate structures as DNA substrates; however, the M oligo also does not match the telomere consensus sequence (30) nor do telomeric sequences match the topoisomerase II consensus sequence (12); furthermore, topoisomerase II does not cleave the telomere sequence *in vitro* (I.K. Chung and M.T. Muller, unpublished observations).

From these data, we conclude that topoisomerase II is reactive towards an unusual four-stranded structure formed by the M oligonucleotide. Furthermore, the activity of topoisomerase II toward this four-stranded structure was unexpected because the enzyme was previously shown not to cleave a polymorphic structure formed by self-association of the oligonucleotide G_{10} (13), and because topoisomerase II sites were shown to be blocked by DNA triplex formation (Chung, Spitzner and Muller, submitted). Thus, the results reported here are novel and uncharacteristic for topoisomerase II.

Other proteins that recognize the MR duplex, such as restriction enzymes, failed to cleave the M4 structure (data not shown). Since topoisomerase II is a homodimer, the tetraplex data suggest that each protomer in the holoenzyme can act independently of the other, as previous work has implied (22). Independent activity of monomers could allow transesterification reactions when monomers in two different enzymes act in trans, leading to recombination (31). Since topoisomerase II can be trapped as a singly nicked intermediate, we proposed (22) that either cleavage or religation is asymmetric or uncoupled and only one protomer is engaged in DNA cutting per enzyme dimer and on a given DNA molecule, the other subunit is idle. Whether or not the idle subunit might be available for reactivity remains to be determined.

An equally interesting interpretation of our finding is that topoisomerase II does not absolutely require a 4 base 5′ overhang between anti-parallel strands for both subunits to be active. The geometry of the bases in the four-stranded DNA molecule might permit topoisomerase II to form a covalent intermediate in which one subunit of a dimer is bound to the 5′ phosphate at site 1 while the other subunit is bound to the 5′ phosphate at site 2. The possibility that the three dimensional structure of DNA may affect how topoisomerase II recognizes and cleaves sites has important implications for the activity of the enzyme in chromatin.

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