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Inhibition of *Mycobacterium smegmatis* topoisomerase I by specific oligonucleotides

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Abstract DNA topoisomerase I from *Mycobacterium smegmatis* unlike many other type I topoisomerases is a site specific DNA binding protein. We have investigated the sequence specific DNA binding characteristics of the enzyme using specific oligonucleotides of varied length. DNA binding, oligonucleotide competition and covalent complex assays show that the substrate length requirement for interaction is much longer (~20 nucleotides) in contrast to short length substrates (eight nucleotides) reported for *Escherichia coli* topoisomerase I and III. P1 nuclease and $KMnO_4$ footprinting experiments indicate a large protected region spanning about 20 nucleotides upstream and 2–3 nucleotides downstream of the cleavage site. Binding characteristics indicate that the enzyme interacts efficiently with both single-stranded and double-stranded substrates containing strong topoisomerase I sites (STS), a unique property not shared by any other type I topoisomerase. The oligonucleotides containing STS effectively inhibit the *M. smegmatis* topoisomerase I DNA relaxation activity.

Key words: DNA topoisomerase I; Mycobacterium; Oligodeoxynucleotide

1. Introduction

Type I DNA topoisomerases [1,2] alter the topology of DNA by transient breakage and resealing of the DNA, while passing the intact strand through the enzyme-operated DNA gate. Type I topoisomerases along with the type II enzymes play a role in maintaining the superhelical density of the cellular DNA [3] and solve the topological constraints in DNA arising due to different macromolecular events [4]. Thus, topoisomerase I has a vital physiological role. Although these enzymes have been studied in great detail from many systems, several questions remain unanswered as to how topoisomerases interact with DNA to carry out their functions in the cell. The type I topoisomerases in general are not considered to be sequence specific DNA binding proteins. However, few members show some degree of sequence specificity [5–7]. Further, in accordance with their function, many of them are known to recognize structures on DNA rather than specific sequences. *Escherichia coli* topoisomerase I and yeast topoisomerase III bind to underwound but not to overwound DNA and hence capable of relaxation of negatively supercoiled DNA. Eukaryotic type I DNA topoisomerases, in contrast, can relax both negatively and positively supercoiled DNA with nearly equal efficiency [8]. Eukaryotic topoisomerases I and II recognize DNA sites possessing intrinsic curvatures [9–11]. Thus, in addition to the conventional classification of topoisomerases as type I and II based on reaction mechanistics, they have been grouped with respect to the conformation of the substrate DNA [12]. The first group includes enzymes which bind to duplex DNA and the second consists of enzymes which preferentially bind to single-stranded DNA [12]. All known type II topoisomerases of both prokaryotic and eukaryotic origin and eukaryotic type I DNA topoisomerases could be considered to belong to the former group; bacterial type I topoisomerase and archaebacterial reverse gyrase would fit into the second group. While the first group of enzymes are known to exhibit a somewhat complex sequence recognition pattern, the cleavage specificity of the latter group is quite simple. The presence of a cytosine four nucleotides away on the 5′-side of the nick (position −4) is observed in majority of the sites mapped for topoisomerase I from *E. coli* [13], *Micrococcus luteus* [14] and archaebacterial reverse gyrase [12]. *E. coli* topoisomerase I can cleave single-stranded oligonucleotides as short as seven bases in length [15]. From these studies, it appears that a normal reaction of this enzyme may involve binding to only a short stretch of DNA [16].

We have characterized DNA topoisomerase I from *Mycobacterium smegmatis*. In many characteristics the enzyme is similar to other prokaryotic type I topoisomerases [17]. However, in contrast to *E. coli* topoisomerase I, fewer cysteines are present in the mycobacterial enzyme and they do not form Zn^{2+} co-ordination for DNA binding [18]. These results indicate a different mode of interaction of the enzyme with DNA and prompted us to study the DNA binding characteristics. We have mapped strong topoisomerase sites (STS) in linear fragments and also in supercoiled plasmids containing genomic sequences from *M. smegmatis* [19,20]. Synthetic oligonucleotides having the consensus sequence were used as substrates for DNA binding and cleavage reactions of the enzyme [20].

In this manuscript, we have carried out detailed studies on site specific interaction of the *M. smegmatis* topoisomerase I with oligonucleotides having the recognition sequence. The enzyme exhibits certain unique features. Efficient interaction with both single- and double-stranded DNA and requirement for larger minimal substrate by the *M. smegmatis* topoisomer-

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Abbreviations: STS, strong topoisomerase site; PAGE, polyacrylamide gel electrophoresis; cpm, counts per minute

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ase I are the key features. In addition, STS containing oligonucleotides inhibit DNA relaxation activity of the enzyme.

2. Materials and methods

2.1. Enzymes and nucleic acids

M. smegmatis topoisomerase I was purified to apparent homogeneity as described previously [18]. Restriction endonucleases and T4 polynucleotide kinase were purchased from Boehringer Mannheim and Gibco BRL. P1 nuclease and the NAP 10 gel filtration columns were obtained from Pharmacia. Supercoiled pUC19 DNA was purified by standard procedures [21]. DNA oligonucleotides were obtained from Bangalore Genei (Pvt.) Ltd., Bangalore, India. Radioisotopes ([γ-^{32}P]ATP and [α-^{32}P]dATP) were purchased from Amersham Corp. Other chemicals used were from Gibco BRL. The oligonucleotides used in this study are as follows: 5′-TATTGGGCGCTCTTCCGCTTCCTCGCTCACTG-3′ (32 mer); 5′-TTGGGCGCTCTTCCGCTTCCTCGCTCAC-3′ (28 mer); 5′-GGGCGCTCTTCCGCTTCCTCGCTC-3′ (24 mer); 5′-GCGCTCTTCCGCTTCCTCGC-3′ (20 mer); 5′-GCTCTTCCGCTTCCTC-3′ (16 mer); 5′-TCTTCCGCTTCC-3′ (12 mer). The respective complementary strands were also used in some of the experiments. Double-stranded oligonucleotides were prepared by mixing equimolar top (oligonucleotide having STS) and bottom (complementary to the top strand) strand oligonucleotides, and then incubating at 90°C for 5 min. The mixtures were then slowly cooled to room temperature. Annealing of the oligonucleotides was assessed by resolving the samples on a native polyacrylamide gel electrophoresis (PAGE) along with the single-stranded oligonucleotides.

2.2. Radiolabeling of oligonucleotides

Oligonucleotides were 5′-end-labeled using [γ-^{32}P]ATP (specific activity, 6000 Ci/mmol) and bacteriophage T4 polynucleotide kinase as per manufacturer's recommendations. The labeled oligonucleotides (specific activity of labeling was approximately 10^8 counts per minute (cpm)/μg of DNA) were purified using Sephadex G-25 spin columns.

2.3. Nuclease P1 protection assay

Reactions (10 μl) contained assay buffer without Mg^{2+} (40 mM Tris–HCl pH 8.0, 20 mM NaCl) and 200 fmol of radiolabeled oligonucleotide. *M. smegmatis* topoisomerase I was added to the above reaction mix and incubated for 5 min at 37°C followed by incubation with 0.003 U of P1 nuclease for 10 min at 37°C. The reactions were terminated by the addition of EDTA to a final concentration of 10 mM, and heat-denatured for 5 min at 90°C after addition of the loading dye (45% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol). The products were separated by electrophoresis through a 15% PAGE.

2.4. $KMnO_4$ footprinting

Oligonucleotides (32 mer top strand, 100 fmol, 10^5 cpm) were incubated with topoisomerase I in 10 μl assay buffer (without Mg^{2+}) on ice for 10 min. Freshly prepared $KMnO_4$ (250 nM final concentration) was added to the reaction mix and incubated at 37°C for 30 s. The reactions were terminated by the addition of 7.5 μl of β-mercaptoethanol (40 mM), 80 μl of sodium acetate (0.375 M) and 50 μg/ml yeast total RNA. The oligonucleotides were ethanol-precipitated twice, washed with 70% ethanol and suspended in 100 μl of 1 M piperidine. Samples were heated at 90°C for 30 min, lyophilized, resuspended in 10 μl of formamide loading dye and analyzed on 15% polyacrylamide–8 M urea gels. The control samples were treated in the same way except that $KMnO_4$ was not added to the reaction mix.

2.5. Electrophoretic mobility shift assay (EMSA)

Non-covalent enzyme–DNA complexes were formed using 40 mM Tris–HCl pH 8.0, 20 mM NaCl, 1 mM EDTA, 10 fmol of radiolabeled oligonucleotide and varying amounts of *M. smegmatis* topoisomerase I by incubating on ice for 5 min. The products were separated through a 5% non-denaturing PAGE (30:0.8). The gels were electrophoresed at 100 V for 1.5 h at 4°C, dried and subjected to autoradiography.

2.6. Gel mobility shift assay for the detection of covalent complex

Radiolabeled 65 bp DNA fragment [19] or different duplex oligonucleotides listed above were incubated with *M. smegmatis* topoisomerase I at 37°C for 15 min. The covalent complexes were arrested by adding 10 mM NaOH. This assay mixture contained 250 fmol of unlabeled oligonucleotide wherever indicated. The products were separated on a 10% PAGE with 0.5×TBE as the running buffer. The gels were electrophoresed at 150 V for 2 h at room temperature, dried and autoradiographed.

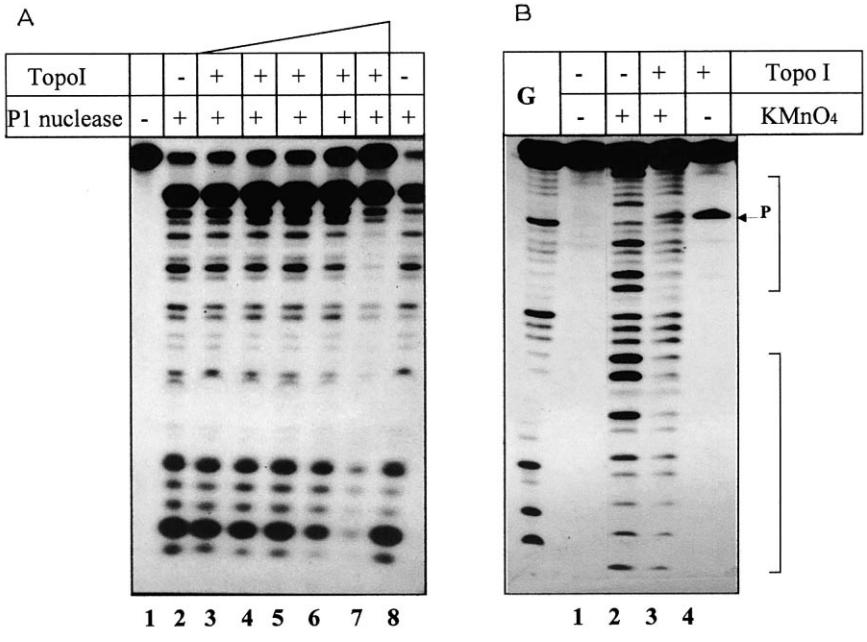

Fig. 1. Topoisomerase I footprinting. Footprinting reactions were performed as described in Section 2. The reaction products were resolved through a 15% polyacrylamide gel containing 8 M urea. A: P1 nuclease protection: substrate DNA alone (lane 1), 0.1 pmol (lane 3), 0.25 pmol (lane 4), 0.5 pmol (lane 5), 1.0 pmol (lane 6) or 2 pmol (lane 7) of topoisomerase I and 0.003 U of P1 nuclease. Lanes 2 and 8 are the P1 nuclease digested (0.003 U) 32 mer oligonucleotide. B: $KMnO_4$ footprint: 32 mer oligonucleotide (lane 1) was treated with 25 nM $KMnO_4$ in absence (lane 2) and in presence (lane 3) of 1 pmol of topoisomerase I. Cleavage product (indicated as P) is obtained from the 32 mer (lane 4). Maxam-Gilbert G reaction of the 32 mer is indicated.

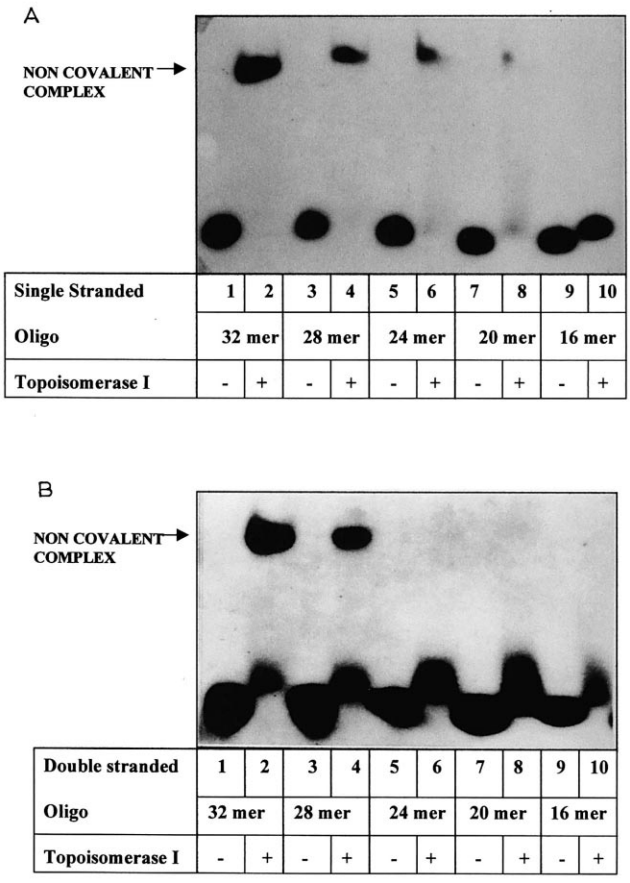

Fig. 2. Binding of topoisomerase I to single- and double-stranded oligonucleotides. EMSA was performed using (A) single- and (B) double-stranded 32, 28, 24, 20 and 16 mer oligonucleotides (100 fmol each). The oligonucleotides were incubated in the absence (lanes 1, 3, 5, 7 and 9) or in 1 pmol (lanes 2, 4, 6, 8 and 10) of topoisomerase I, respectively. The complexes were resolved through a 5% polyacrylamide gel at 4°C. The position of the topoisomerase I–oligonucleotide non-covalent complex is indicated.

2.7. DNA relaxation assay

The reaction mixture contained 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 20 mM NaCl, 0.33 pmol of pUC19 negatively supercoiled DNA and 1 U of *M. smegmatis* topoisomerase I. Reaction mixes were incubated at 37°C for 15 min, stopped with 0.5% sodium dodecyl sulfate and samples were separated on a 1.2% agarose gel. The DNA was visualized by ethidium bromide staining.

3. Results

3.1. Topoisomerase I footprint on substrate DNA

The interaction of *M. smegmatis* topoisomerase I with its binding site was analyzed by footprinting experiments using 32 mer oligonucleotides. Footprinting reactions were carried out using P1 nuclease and $KMnO_4$ as probes (Fig. 1A,B). With higher amounts of topoisomerase, a large protected region was observed when probed with P1 nuclease. The enzyme protects almost the entire 32 mer oligonucleotide (Fig. 1A, lane 7). The results with $KMnO_4$ footprinting show two protected regions which are located within the P1 nuclease footprint (Fig. 1B). These results demonstrate that the enzyme binds a large stretch of DNA wherein cleavage site is located towards the end of the protected region.

3.2. Minimal substrate required for binding

In order to assess the minimal length of DNA required for topoisomerase I binding, STS containing oligonucleotides of varied length were used (see Section 2). Binding was assessed by the formation of the non-covalent complex between the topoisomerase I and oligonucleotides and resolved by a native PAGE (Fig. 2A,B). Non-covalent binding decreases with the decreasing length of the oligonucleotides for both single- and double-stranded substrates. While the 32, 28, 24 and 20 mer single-stranded oligonucleotides (top strand) form the enzyme–DNA complex, only the 32 mer and 28 mer duplex oligonucleotides show the complex formation. The inefficient binding of the enzyme to shorter oligonucleotides could be due to rapid dissociation of the enzyme as a result of insufficient contacts with DNA in agreement with footprinting results. Single-stranded oligonucleotides having STS seem to be relatively better substrates for enzyme binding. Bottom strand oligonucleotides (oligonucleotides without STS sequence) do not form such complexes reflecting the importance of the presence of STS.

3.3. Covalent complex formation with duplex oligonucleotides

Ability of the enzyme to interact with the substrate was assessed by arresting the DNA–protein covalent intermediate of the reaction. This experiment would also reveal the minimum length of the double-stranded DNA required for the first transesterification reaction. The 32 and 28 mer duplex oligonucleotides formed covalent complexes under the reaction conditions (Fig. 3). The complex formation is weaker with 28 mer and hardly detectable with 24 mer. No detectable complex was observed with the smaller duplex oligonucleotides (Fig. 3).

3.4. Competition assay with the top and bottom strand oligonucleotides

Minimum substrate length requirement and site specific interaction of the enzyme was demonstrated by a competition assay. An end-filled 65 bp fragment of pUC19 having STS for the *M. smegmatis* topoisomerase I [19] was used as a substrate for covalent complex formation. Unlabeled top strand or bottom strand oligonucleotides of different sizes were used as

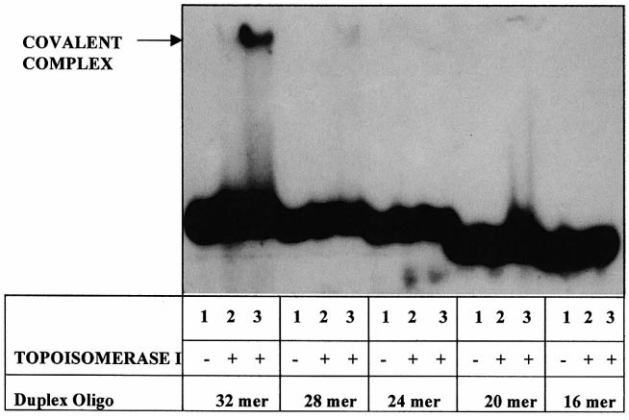

Fig. 3. Minimum substrate length required for covalent complex formation by *M. smegmatis* topoisomerase I. Double-stranded oligonucleotides were incubated in the absence (lane 1) or in 1 pmol (lane 2) and 2 pmol (lane 3) of topoisomerase I and complexes were trapped with alkali. The samples were processed as described in Section 2. Lengths of the oligonucleotides are indicated.

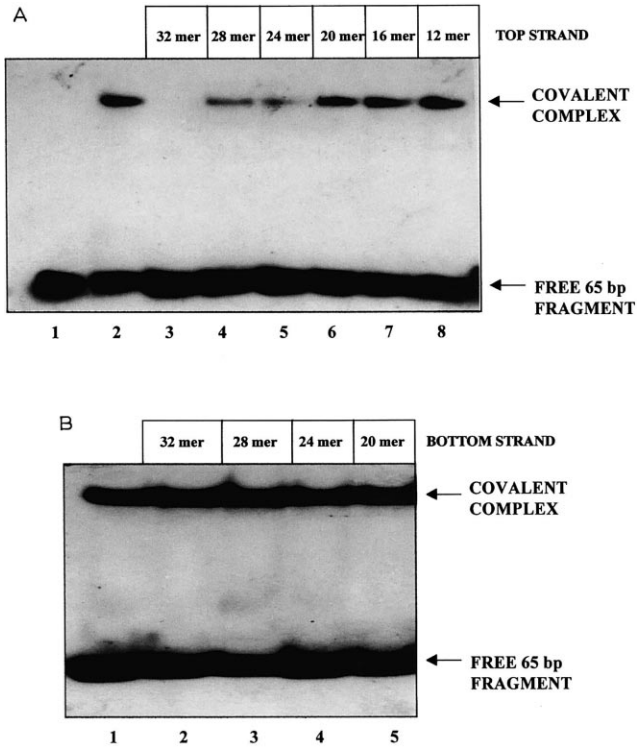

Fig. 4. Competition for covalent complex formation. End-labeled 65 bp DNA fragment (100 fmol) was used as the substrate for covalent complex formation with 1 pmol of topoisomerase I and the complex was arrested with NaOH as described earlier [19] and in Section 2. A: Competition assay with top strand oligonucleotides. 1 pmol of unlabeled 32, 28, 24, 20, 16 and 12 mer oligonucleotides containing STS were used as competitors (lanes 3–8). Lane 1: no enzyme, lane 2: no competitor. B: Effect of bottom strand oligonucleotides. Lane 1: no competitor, 1.0 pmol of unlabeled 32, 28, 24 and 20 mer oligonucleotides lacking the STS (lanes 2–5) were used as competitors, respectively.

competitor substrates. Amongst the top strand oligonucleotides, the 32 mer competes effectively with the 65 bp fragment for complex formation (Fig. 4A, lane 3). Competition is also seen with the 28 and 24 mers, albeit to a lesser extent. In contrast, the 20, 16 and 12 mer oligonucleotides having STS do not seem to affect considerably the covalent complex formation. When the bottom strand oligonucleotides were used as competitors, complex formation was unaffected irrespective of the size of the oligonucleotides (Fig. 4B, lanes 2–5). These results highlight the importance of presence of STS in the oligonucleotides as well as the requirement of minimal substrate length for the interaction of topoisomerase I with DNA.

3.5. *Inhibition of DNA relaxation activity of topoisomerase I by oligonucleotides*

Above results indicate that oligonucleotides containing STS should be effective inhibitors for overall reaction of the enzyme. This is directly demonstrated by using the top strand oligonucleotides containing STS as inhibitors of topoisomerase I catalyzed relaxation of negatively supercoiled DNA. A representative set of data is shown in Fig. 5. Both 32 mer and 28 mer oligonucleotides inhibit the DNA relaxation activity in a 1:0.5 molar ratio (plasmid:oligonucleotide). In contrast, the 32 mer complementary strand oligonucleotide (without the STS) does not inhibit the relaxation activity of the enzyme even at a 100-fold excess concentration (Fig. 5B, lane 12). The summary of the DNA relaxation inhibition data is presented in Fig. 5C. With shorter oligonucleotides, higher concentrations were required for inhibition of the DNA relaxation activity of the enzyme. These results are in agreement with the competition, binding and covalent complex assays. The above experiments while demonstrating the effectiveness of the oligonucleotides as inhibitors, further substantiate the minimal substrate length requirement for the enzyme.

4. Discussion

We have probed the site specific interaction of *M. smegmatis* topoisomerase I at its cognate site. Minimum substrate length needed for binding and subsequent reaction is determined which is considerably larger amongst all known type IA topoisomerases. Further, oligonucleotides having STS were shown to inhibit DNA relaxation activity of the enzyme.

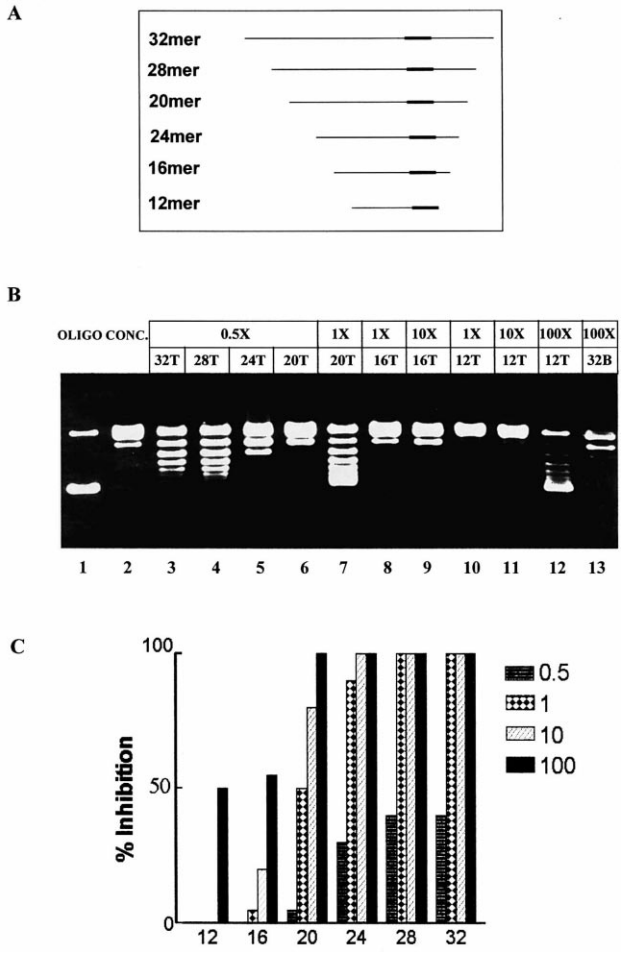

Fig. 5. Inhibition of topoisomerase I catalyzed DNA relaxation by the STS containing oligonucleotides. A: The oligonucleotides (see Section 2) used for assessing the inhibition of relaxation activity are illustrated. Thick line represents the position of STS within the oligonucleotides. B: DNA relaxation activity: DNA relaxation assay was carried out using pUC19 DNA (0.33 pmol) with 2 ng of topoisomerase I and reaction products were resolved as described in Section 2. Lane 1 is supercoiled pUC19 and lane 2 shows the relaxation reaction. The length of the oligonucleotides and the molar ratios with respect to pUC19 are indicated. T and B refer to top and bottom strands, respectively. C: Summary of inhibition data: percentage inhibition of relaxation reaction by the oligonucleotides is depicted.

E. coli topoisomerase I or III were shown to efficiently react with 7–8 nucleotide long single-stranded oligonucleotides [15,22] while even a 20 mer is a relatively poor substrate for the enzyme from *M. smegmatis*. These results suggest an elaborate, more complex interaction of the enzyme with the substrate DNA. Large protected region in footprint analysis supports this view. Inability of smaller oligonucleotides to inhibit relaxation activity together with other experiments presented in this report further substantiate this point. Another important property of the mycobacterial topoisomerase emerging from these studies is the efficient interaction of the enzyme with both single- and double-stranded oligonucleotides having recognition sequences. *E. coli* topoisomerase III [22] is known to interact site specifically with single-stranded DNA. In contrast, eukaryotic enzymes (type IB) recognize duplex DNA as substrates [23,24]. It would be of interest to decipher the molecular basis of distinct recognition pattern exhibited by *M. smegmatis* enzyme.

Site specific, high affinity DNA binding proteins generally recognize symmetric sequences. However, the STS of *M. smegmatis* enzyme does not show any symmetry. The enzyme's mode of interaction could be asymmetric as well. The data with different length DNA substrates and $KMnO_4$ footprinting indicate that the enzyme interacts asymmetrically with DNA relative to its site of cleavage. The cleavage site is located towards the 3′-end of the protected region. The asymmetric sequence recognition pattern of the enzyme is similar to that of topoisomerase III of *E. coli* [22].

A parallel can be drawn in the behavior of *M. smegmatis* and human topoisomerase I in this regard. Human topoisomerase I is also a site specific DNA binding protein and its activity has been shown to be inhibited by specific oligonucleotides [25]. Efficient, specific inhibition of the enzyme activity is one of the remarkable features presented in this paper. Only the oligonucleotides having the certain length and containing recognition sequence serve as effective inhibitors of the enzyme. The results indicate that the oligonucleotides bind and squelch away the enzyme molecules essentially denying access to the supercoiled substrates.

Thus, the interaction of *M. smegmatis* topoisomerase I at its STS has certain distinctive features. While the unique characteristics reflect the functional requirement, they open up avenues to address reaction mechanistics and explore therapeutic potential. Although topoisomerase I from prokaryotes plays a central role in maintaining homeostatic balance of superhelicity of the genome and thus influences the essential functions, no inhibitor for the enzyme has been identified. Detailed structure–function studies with both the enzyme and the substrate DNA could form the basis for the design of inhibitors for therapeutic and scientific applications.

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