

# A Novel Bipartite Mode of Binding of *M. smegmatis* Topoisomerase I to its Recognition Sequence

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We have investigated interaction of *Mycobacterium smegmatis* topoisomerase I at its specific recognition sequence. DNase I footprinting demonstrates a large region of protection on both the scissile and non-scissile strands of DNA. Methylation protection and interference analyses reveal base-specific contacts within the recognition sequence. Missing contact analyses reveal additional interactions with the residues in both single and double-stranded DNA, and hence underline the role for the functional groups associated with those bases. These interactions are supplemented by phosphate contacts in the scissile strand. Conformation specific probes reveal protein-induced structural distortion of the DNA helix at the T-A-T-A sequence 11 bp upstream to the recognition sequence. Based on these footprinting analyses that define parameters of topoisomerase I-DNA interactions, a model of topoisomerase I binding to its substrate is presented. Within the large protected region of 30 bp, the enzyme makes direct contact at two locations in the scissile strand, one around the cleavage site and the other 8-12 bases upstream. Thus the enzyme makes asymmetric recognition of DNA and could carry out DNA relaxation by either of the two proposed mechanisms: enzyme bridged and restricted rotation.

**Keywords:** footprinting; mycobacteria; Sequence specificity; Topoisomerase I

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## Introduction

The topological state of the DNA is known to play crucial roles in vital cellular processes by modulating the flow of information encoded in the double helix. Topoisomerases are enzymes that catalyze topological inter-conversion to relieve the torsional strain introduced in the double helix by cellular processes such as replication, transcription, recombination, repair and chromosome segregation.<sup>1</sup> Mechanistically, DNA topoisomerases are classified into two groups based on their mode of DNA strand scission: the type I enzymes introduce single-stranded nicks, whereas the type II topoisomerases mediate transient breakage of the sugar phosphate chains at both the strands of DNA substrate. Subsequent to the cleavage and strand passage, religation of the phosphodiester bond occurs, changing the linking number by either steps of one (type I) or two (type II).<sup>2,3</sup> The

type I enzymes are further categorized into two groups, IA and IB, based on the biochemical properties and the type of reaction intermediate. Members of type IA form a 5' phospho-tyrosine adduct during the first trans-esterification reaction, and generally require a single-stranded region in the DNA and magnesium as a cofactor in a reaction restricted to relaxation of the negatively supercoiled DNA. Examples include *Escherichia coli* topoisomerase I and topoisomerase III, *Bacillus subtilis* topoisomerase I and reverse gyrase.<sup>4,5</sup> Type IB enzymes, in contrast, form a covalent contact with the 3' residue during the first catalytic step of trans-esterification. Members of this subclass utilize duplex DNA as substrate and are able to act on both positively and negatively supercoiled substrates in a metal ion-independent DNA relaxation reaction. Examples of this group include nuclear topoisomerase I found in all eukaryotic cells, topoisomerase V, and the topoisomerase I encoded by the vaccinia and other cytoplasmic viruses.<sup>4</sup>

To gain insight into the function and reaction mechanism, attempts have been made to study the interaction of topoisomerase with substrate DNA.<sup>6-11</sup> The data obtained from various sys-

Abbreviations used: STS, strong topoisomerase site; SS, scissile strand.

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tems suggest that the sequence specificity of topoisomerases is not very stringent although they appear to prefer certain sequences to others.<sup>7,12-18</sup> Topoisomerase I from vaccinia virus is perhaps the only exception, which is distinctive in its site specificity.<sup>8</sup> Detailed studies have been carried out on the site specific interaction of vaccinia topoisomerase I with DNA and as a result the viral protein has emerged as the model for understanding the reaction mechanism of type IB topoisomerases.<sup>19</sup> The biochemical knowledge on the enzyme was complemented by the structural information of the viral topoisomerase I.<sup>20,21</sup> These studies have led to the proposal of free-rotation model of relaxation for the enzyme.<sup>22</sup> Meanwhile, mechanistic studies with human topoisomerase I-DNA complexes provided insights into the structural features important for the interaction of the latter enzyme, leading to a different scheme of DNA relaxation.<sup>23,24</sup>

Three-dimensional structure of the truncated *E. coli* topoisomerase I<sup>9</sup> provided data about the domains of the protein, and suggested a probable relaxation mechanism. Based on these studies, the members of type IA subclass are believed to effect DNA relaxation by a enzyme-bridged mechanism.<sup>9</sup> However, unlike the vaccinia topoisomerase, *E. coli* topoisomerase I does not exhibit high degree of sequence specificity, and hence only limited information on interaction with substrate and reaction mechanism is available. In this manuscript, we have taken the advantage of site-specific binding of *Mycobacterium smegmatis* topoisomerase I to address the possible reaction mechanism of type IA topoisomerases. *M. smegmatis* topoisomerase I has several unusual features. While the protein exhibits characteristics akin to type IA topoisomerases it lacks the zinc-finger motif, found in other members of type IA family.<sup>25</sup> This is the only known topoisomerase that interacts with both single and double-stranded DNA with comparable affinity,<sup>26</sup> and displays a high degree of sequence specificity.<sup>26,27</sup> These features of topoisomerase I make it a suitable candidate to address the molecular basis of site-specific interaction. Here, we describe the interaction pattern of *M. smegmatis* topoisomerase I with DNA. The novel footprinting pattern differs from any other topoisomerase I characterized so far, and provides clues into the reaction mechanism.

## Results

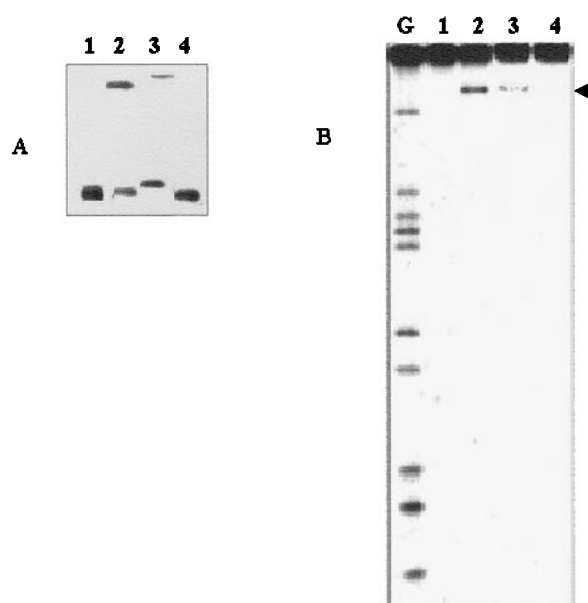
### *M. smegmatis* topoisomerase I interacts with DNA in a sequence specific manner

Mycobacterial topoisomerase I displays remarkable sequence specificity in recognizing the DNA sites containing the CGCTTC/G element.<sup>26,27</sup> A 38mer oligonucleotide harboring this strong topoisomerase site (STS) was used in all footprinting experiments (see Experimental Procedures).

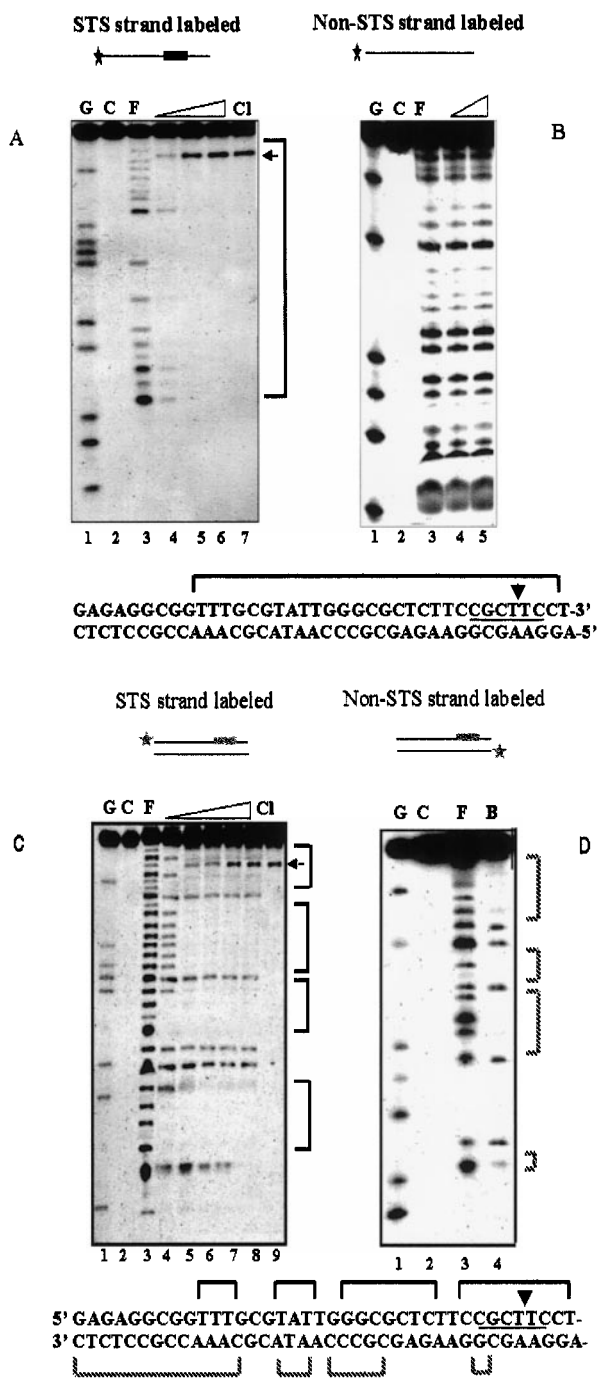
Incubation of topoisomerase I with STS containing oligonucleotides resulted in a distinct topoisomerase-DNA complex (Figure 1(a), lanes 1 and 2). The absence of such a retarded complex upon incubating the protein with non-specific oligonucleotides under identical conditions (Figure 1(a), lane 4) demonstrates the specificity of the interaction of the enzyme to the DNA having recognition sequence. Similar results were obtained in topoisomerase-mediated cleavage reactions of single and double-stranded DNA (Figure 1(b), lanes 2 and 3). No cleavage product was detected when non-specific oligonucleotides were used (Figure 1(b), lane 4).

### DNase I footprinting

DNase I footprinting assays provide overall protection pattern showing the surface area of DNA occupied by the protein. The end-labeled



**Figure 1.** Characterization of the substrate DNA used in footprinting reaction. (a) Specific binding of topoisomerase I: oligonucleotides containing the STS (lanes 1-3) or lacking it (lane 4) were incubated either in the absence (lane 1) or in the presence of 1 ng of *M. smegmatis* topoisomerase I (lanes 2 and 3) and then electrophoresed through a native 8% polyacrylamide gel at 150 V for one hour. In lane 3, STS DNA in double-strand context was used. (b) Specific cleavage of the STS oligonucleotide: G ladder (Lane G); free DNA (lane 1). Topoisomerase was incubated along with STS oligonucleotide (10 fmol) in single (lane 2) or double-stranded context (lane 3), or incubated with oligonucleotide lacking STS (lane 4). The reaction products were electrophoresed through a denaturing 16% polyacrylamide gel and exposed to autoradiography. The arrow indicates the topoisomerase cleavage site.



**Figure 2.** DNase I protection analysis. (a) Footprinting on single-stranded DNA 0.1 pmol of STS (represented by filled box) oligonucleotide was used in the assay. G ladder (lane 1); substrate DNA (C; lane 2), either treated with 5 ng of DNase I alone (F; lane 3) or incubated with varying concentrations of topoisomerase I (0.5, 1 and 5 ng) prior to the DNase I treatment (lanes 4-6, respectively). Topoisomerase I (10 ng) cleavage product obtained by incubating it with the substrate is indicated by an arrow (CI; Lane 7). (b) Footprinting on non-STS oligonucleotides. G ladder (lane 1); labeled DNA (C; lane 2), was either incubated with DNase I alone (F; lane 3) or incubated with 5 ng (lane 4) or 10 ng (lane 5) of topoisomerase I and then treated with 5 ng of DNase I. (c) Footprinting on double-stranded DNA. Labeled STS DNA in double-stranded context was used. G lad-

der (lane 1); STS DNA, untreated (C; lane 2), pre-incubated either in the absence (F; lane 3) or in the presence of 1, 2.5, 5, 10 and 15 ng of topoisomerase I (lanes 4-8, respectively) and then treated with 5 ng of DNase I. Topoisomerase I (15 ng) mediated cleavage reaction is indicated by an arrow (CI; lane 9). (d) Labeled non-STS oligonucleotide in double-stranded context was used for the assay. G ladder (lane 1); the duplex DNA, untreated (C; lane 2), pre-incubated either in the absence (F; lane 3) or in the presence (B; lane 4) of topoisomerase I (10 ng) and then subjected to DNase I digestion. In all the cases the reaction products were resolved through 16% urea-PAGE. Parenthesis marks the region of protection and is indicated in the sequence below. The sequence underlined represents the STS.

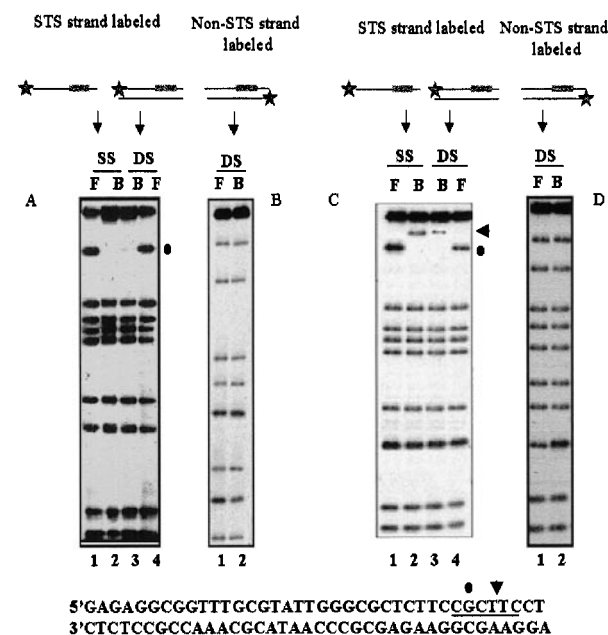
## Base-specific recognition by mycobacterial topoisomerase I

### *Topoisomerase I contacts a G residue within the STS*

One of the ways of target recognition by site-specific DNA-binding proteins is by direct contact with the residues in the major and/or minor groove, enabling discrimination of specific sequence from the unrelated ones. In order to map the residues, which are in close proximity of the protein, DMS protection and interference assays were carried out. The reagent methylates the N-7 position of guanine bases in the major groove and N-3 of adenine bases in the minor groove.<sup>29</sup> Comparison of piperidine cleavage products following DMS treatment revealed protection of a guanine

residue present within the recognition sequence (Figure 3(a), lane 2). The results were identical in double-stranded context as well (Figure 3(a), lane 3). The single G residue present in the minimal recognition site was protected (Figure 3(a) lanes 2 and 3). No additional G residue contacts could be detected when duplex DNA having the label in non-scissile strand was used as a substrate (Figure 3(b)).

Binding of topoisomerase I was also assayed following methylation of the oligonucleotide substrate in single strand context. DNA corresponding to the free and bound fractions were separated and analyzed after piperidine cleavage. Comparison of the cleavage products revealed the near complete exclusion of the piperidine cleavable site corresponding to the -3G in the scissile strand from the bound fraction (Figure 3(c), lane 2). In contrast, methylation of G residues, situated elsewhere did not affect the binding. The pattern of methylation interference was similar in double-stranded DNA (Figure 3(c), lane 3) to results obtained with single strand DNA (Figure 3(c), lane 2). A cleavage product of the radiolabeled scissile strand was detected in the protein-bound DNA fraction, but not in the free DNA population (Figure 3(c), lane B). The G residue protected (Figure 3(a)) from chemical modification was the same residue whose pre-modification interfered with DNA binding. No interactions were detected with the non-scissile strand in double-stranded context (Figure 3(d)).

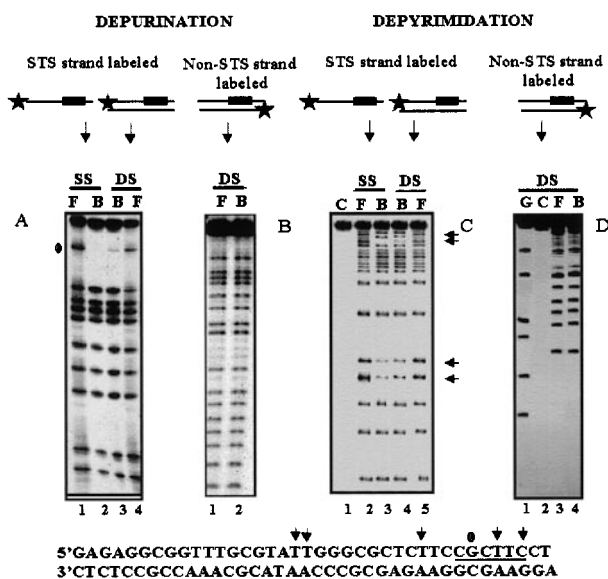


**Figure 3.** DMS reactions: methylation protection (a) Topoisomerase I (150 ng) was incubated with STS oligonucleotide in single (lane 2) or double-stranded context (lane 3) and subjected to DMS modification. SS indicates scissile strand in single-stranded context (lanes 1 and 2) while DS represents the double-strand context (lanes 3 and 4). (b) Labeled non-STS DNA was incubated with topoisomerase I (300 ng) in double-stranded (DS) context prior to methylation by DMS. Methylation interference (c) Labeled STS oligonucleotide was pre-methylated and incubated with topoisomerase I (150 ng). DNA corresponding to free (lane F) and DNA-protein complex (lane B) are indicated. Topoisomerase mediated cleavage is indicated by an arrow. (d) Labeled non-STS DNA was pre-methylated, hybridized to unlabeled STS strand and incubated with topoisomerase I (300 ng). In all the panels, F and B represent the free and bound fractions, respectively. Filled boxes represent the STS while the filled circles indicate the G contact. Topoisomerase induced cleavage is indicated by an arrow, while the STS sequence is underlined.

### *Additional base contacts of topoisomerase I revealed by missing contact experiments*

As the previous set of experiments revealed only a single base specific contact and DNase I footprinting showed a large protected region, we investigated whether there were any additional interactions. Moreover, a single hydrogen bond is unlikely to be the sole determinant of the sequence specific recognition.<sup>30</sup> In addition, our previous studies with the STS-containing synthetic oligonucleotides having different base substituents revealed the importance of +1 and +2 residues.<sup>27</sup> In order to identify the contribution of other nucleotides in high-specificity interaction of the topoisomerase I, missing nucleoside interference analyses were performed.<sup>31,32</sup> Protein was incubated with the modified DNA and the bound fractions were separated as described in experimental procedures. Depurination of the -3G residue within the STS interfered with the topoisomerase I binding (Figure 4(a), lane 2). This is the same G residue shown to interact with enzyme by DMS protection and interference assays. In contrast, purines located at the immediate upstream of the minimal motif (CGCTTC/G; the recognition sequence for the site-specific binding of topoisomerase I<sup>26,27</sup>) do not seem to be in close proximity to the protein (Figure 4(a) lane 2). Similar results were obtained in double-stranded context





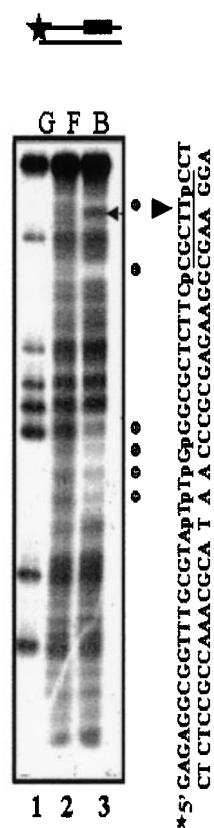
**Figure 4.** Missing base contacts experiments: depurination (a) The labeled scissile strand was modified using formic acid and incubated with 150 ng of topoisomerase I. DNA corresponding to free (F; lane 1) and bound fractions (B; lane 2) are indicated. STS strand was depurinated, annealed to the unlabeled bottom strand and then incubated with 300 ng of topoisomerase I. The bound (B; lane 3) or free (F; lane 4) fractions are indicated. (b) Labeled non-STS strand was depurinated, annealed to the unlabeled STS strand and then incubated with 300 ng of topoisomerase I. The free (F) and bound (B) fractions (lanes 1 and 2, respectively) are shown. Depyrimidation (c) The labeled STS strand (C; lane 1) was modified by hydrazine and incubated with 150 ng of topoisomerase I (lanes 2 and 3) or annealed to non-STS strand (lanes 4 and 5) prior to incubation with 300 ng of topoisomerase I. The free (F; lanes 2 and 5) and bound (B; lanes 3 and 4) fractions are indicated. (d) Labeled non-STS DNA was depyrimidated, annealed to the unlabeled scissile strand and used for the assay. G ladder (lane 1); the substrate DNA (C; lane 2) was incubated with 300 ng of topoisomerase I. The free (F; lane 3) and bound (B; lane 4) fractions indicated. The purine contact is represented by the filled circle while the arrows indicate the pyrimidine contacts. The sequence underlined represents the STS.

(Figure 4(a) lane 3). Purine residues in the bottom strand do not seem to be contacted by the protein (Figure 4(b)).

Depyrimidation experiments revealed the important pyrimidine contacts of the protein (Figure 4(c), lanes 2 and 3). These base contacts are localized in two distinct regions in the DNA. Two of these contacts were with +2C and -1T within the STS whereas other critical contacts were with -7T, -16T and -17T residues, upstream to the STS. Pyrimidine-specific contacts were not observed with bottom strand when annealed to the scissile strand (Figure 4(d)).

## Electrostatic interactions with DNA backbone of the scissile strand

Apart from the base-specific contacts, ionic interactions between the DNA and protein contribute towards the energy required for binding. In addition, sequence-specific binding is enhanced by DNA backbone contacts with the protein as they govern the geometry between protein and DNA.<sup>33</sup> Phosphate contacts made by mycobacterial topoisomerase I were determined using ethylnitrosourea-modified substrate DNA. The footprinting pattern obtained is shown in Figure 5 depicting the comparison of the distribution of products in bound and free fractions. Each fragment moves as a doublet representing heterogeneous population of 3'-hydroxyl and 3'-ethylphosphates.<sup>32</sup> As can be seen in Figure 5, the strongest inhibition to binding

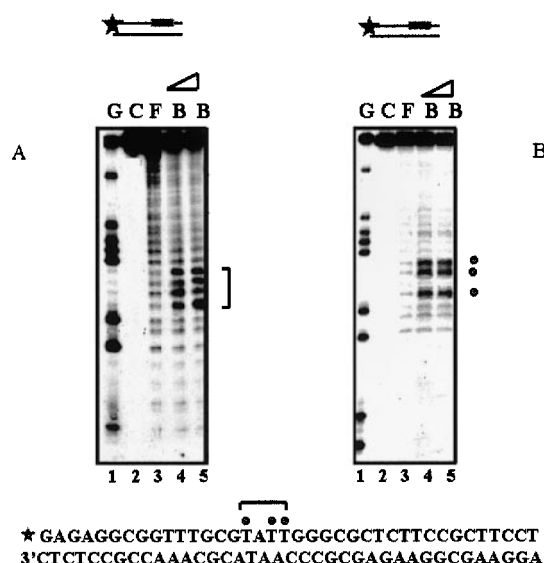


**Figure 5.** Phosphate contacts. STS DNA was modified by ethylnitrosourea, and incubated with 100 ng topoisomerase I. The DNA-protein complex was separated from the free DNA on a native 8% polyacrylamide gel, purified and cleaved at the modified sites, as described in Experimental Procedures. G ladder (lane 1); free DNA (F; lane 2); bound fraction (B, lane 3). STS is marked by filled box. The phosphate contacts are indicated by filled circles and represented by "p" in the sequence. Topoisomerase cleavage site is indicated by an arrow, while the STS is represented by the underlined sequence.

occurs when the two phosphates (p) in the sequence TCpCGCTTpC are ethylated. The sites of complete interference therefore correspond to important contacts between the DNA and the protein. Ethylation at other sites inhibits the complex formation to varying degrees. When the contiguous phosphates upstream to the STS in the sequence 5'-TApTpTpGpG are modified, the binding is partly inhibited. Thus interaction with six sugar phosphates seems to be important for DNA binding, five of which are localized upstream to the recognition sequence, and only one is within the STS. No phosphate contacts are established with the bottom strand (not shown).

### Change in the B-DNA conformation of substrate DNA

Although the enzyme also binds duplex DNA efficiently and protects it from nuclease digestion, base- and backbone-probing experiments revealed contacts only in the scissile strand. To better understand the interaction, chemical nuclease, 1,10 phenanthroline-copper, a redox-active coordination complex that nicks the phosphodiester backbone of DNA by oxidative degradation of the deoxyribose moiety, was used. Unlike the DNase I protection, Cu(OP)<sub>2</sub> probing did not result in protection of the recognition sequence (Figure 6(a)). Similar observations were made with *Bsp*RI methyl transferase; while DNase I footprinting revealed a protected region, Cu(OP)<sub>2</sub> failed to show any protection with *Bsp*RI.<sup>34</sup> DNase I, interacts with both the major and minor groove,<sup>35</sup> whereas the chemical nuclease is known to bind only to the minor groove.<sup>35</sup> Thus, the results depicted in Figure 6(a) suggest that the protein either does not make any minor groove contact or even if it does, it does not alter the conformation of the minor groove so as to block the accessibility of 1,10 phenanthroline-copper to hydrogen. A stretch of four residues at the positions -17 to -20 (Figure 6(a)), upstream to cleavage site revealed enhanced reactivity to Cu(OP)<sub>2</sub> in spite of lack of intrinsic preference of the chemical nuclease for A and T residues.<sup>36</sup> The hyper-reactivity of these residues reflects protein-induced alteration of DNA conformation. Additional support for the distortion was obtained by conformational mapping of the substrates with KMnO<sub>4</sub>, which selectively modifies the unstacked thymidine residues. Since three of the four hyperactive residues observed in Figure 6(a) happen to be T, it should be reactive to the KMnO<sub>4</sub>, provided these residues exist in unstacked conformation. Figure 6(b) demonstrates the structural deformity of DNA at these T residues. A point to be noted is that two of these T residues were shown to be important for protein binding, as revealed by missing nucleoside experiments (see Figure 4(c)).



**Figure 6.** Structural distortion in DNA. STS oligonucleotide annealed to the non-STS DNA was used for the assays. The reaction products were resolved on a denaturing 18% polyacrylamide gel and autoradiographed. (a) Cu(OP)<sub>2</sub> footprinting. G ladder (lane 1), substrate DNA (C; lane 2), was treated with Cu(OP)<sub>2</sub> in the absence (F; lane 3) or presence of 100 and 200 ng of topoisomerase I (B; lanes 4 and 5, respectively). Parenthesis indicates the hypersites. (b) KMnO<sub>4</sub> footprinting. G ladder (lane 1); substrate DNA (C; lane 2), incubated either in the absence (F; lane 3) or in the presence of 50 (B; lane 4) and 100 ng (B; lane 5) of topoisomerase and then probed with KMnO<sub>4</sub>. Filled circles correspond to the hyper-reactive residues and are indicated in the sequence below.

## Discussion

Footprinting analyses probing the *M. smegmatis* topoisomerase I-DNA complex, enabled us to determine the different types of contacts responsible for specific interactions. The recognition pattern of the enzyme has novel features not seen so far for any other topoisomerase, providing insights into the reaction mechanism. From the overall interaction pattern it appears that the enzyme employs an enzyme-bridged mechanism to remove negative supercoils from DNA.

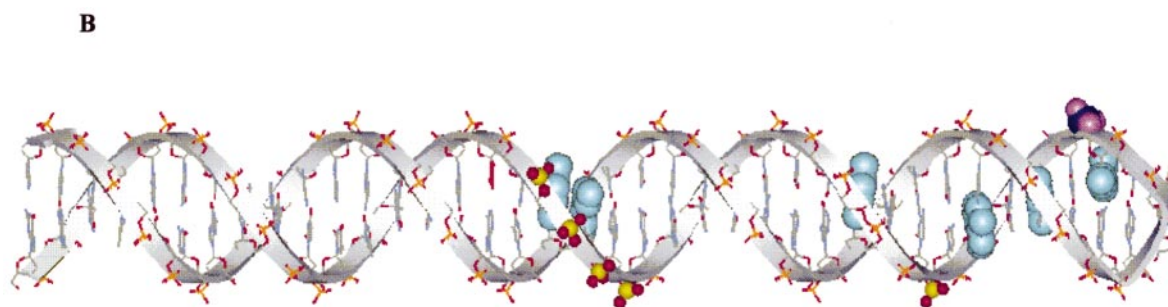
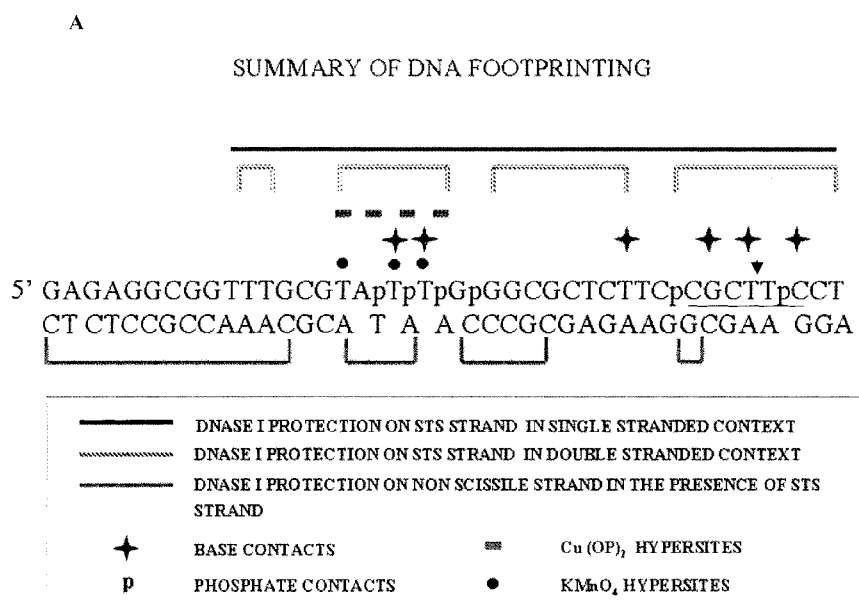
### DNA binding of mycobacterial topoisomerase I

The DNase I footprint of mycobacterial topoisomerase I spans a region of about 30 bases in both single and double-stranded context suggesting a large surface area of interaction. In contrast, *E. coli* topoisomerase III, which is known to show some sequence specificity protects a region of 14 nucleotides in single-stranded DNA.<sup>37</sup> However, other members of 1A group, *E. coli* and *Micrococcal luteus* topoisomerase I, do not form

clear footprints, and are shown to protect a region of more than 50 nucleotides on both the strands of DNA, indicating the binding of several topoisomerase molecules.<sup>38</sup> It is noteworthy that both *E. coli* and *M. luteus* enzymes do not exhibit remarkable sequence specificity unlike the former two enzymes.

The base-specific and phosphate contacts of mycobacterial topoisomerase is bipartite and restricted to the scissile strand (Figure 7). The six hydrogen-mediated base contacts established by the protein involves a lone purine contact at -3G and five pyrimidines +2C, -1T, -7T, -16T and -17T. Three of these base-specific interactions are within the STS, namely, +2C, -1T and -3G. From the phosphate interference assay it appears that six electrostatic contacts are important for protein-DNA interaction, one of which is within the STS between the +1T and +2C (Figure 7). Though interactions of mycobacterial topoisomerase I map on both the faces of the helix, the protein contacts

only the top strand (Figure 7). Thus, mycobacterial topoisomerase is intrinsically a single-strand DNA-binding protein, with the ability to recognize the scissile strand in duplex context, as well with a marginally reduced affinity (Figures 1(b), 3(a), 4(a)). The *E. coli* MutL protein involved in DNA repair exhibits similar binding features wherein affinity for single-stranded DNA is twofold higher than for double-stranded DNA.<sup>39</sup> These results are expected as single-stranded DNA is susceptible to a variety of interactions between the phosphate backbone, surrounding water molecules and the cations, it can adopt several conformations. In contrast, determinants that shape the helical geometry, i.e. the stacking interactions and the inter-phosphate repulsion,<sup>40,41</sup> impose restrictions on the movement of the sugar phosphate backbone, which in turn affect the flexibility of the bases in the double-stranded context.<sup>42</sup> Additionally, the functional groups of the bases in double-stranded DNA are less accessible owing to a constellation of



**Figure 7.** (a) Summary of Footprinting data. The sequence underlined represents the STS and the arrow indicates the cleavage site. (b) Modeling of the substrate DNA: The 38mer substrate DNA was modeled using Molscript and visualized in Weblab viewer Lite 3.5. Base specific contacts (in cyan) and phosphate contacts (in yellow) are shown. The scissile phosphate is indicated (in blue).

atoms from sugar and the complementary bases. Interactions between the charged, as well as uncharged molecules, and the van der Waals repulsion from the neighboring atoms both in plane and out of plane could restrict free ligand binding. The higher degree of flexibility and easier accessibility of the functional groups in single-stranded DNA could attribute to the marginal differences in the contact pattern of the enzyme in single and double-stranded context.

### Structural distortion of DNA

Although the DNA determinants of enzyme contact lie in the scissile strand, the enzyme exhibits a remarkable ability to interact with double-stranded DNA. This possibly stems from the property of the enzyme to distort double-stranded DNA after binding. The mycobacterial topoisomerase I induces localized structural distortion upstream to STS.  $\text{KMnO}_4$  probing suggests the formation of single-strand region at the T-T-A-T stretch (17 to -20), which exhibits hypersensitive cleavage. The strong hold on the scissile strand by the enzyme within the same sequence is reflected by the base-specific contacts with two of these residues apart from the four phosphate contacts. Thus, region upstream to the cleavage site, seems to play a crucial role in defining the geometry of the protein-DNA interface, and may enhance the sequence-specific binding. Phosphate contacts, as well as conformational change induced by protein-DNA phosphate contacts, have been implicated to aid the process of sequence recognition.<sup>33</sup>

### Modeling the DNA

Summary of different footprinting results presented in Figure 7(a) is projected as a molecular model of the DNA contact sites of *M. smegmatis* topoisomerase I (Figure 7(b)). Analysis of the DNA contacts by the enzyme in the context of B-DNA model, reveals important features. The protein interacts with its substrate DNA on both the sides of double helix as the protein-base and protein-phosphate contacts are arrayed across the double helix. This mode of interaction is reminiscent of the DNA interaction pattern of topoisomerases wherein the protein clamps the DNA within the centrally placed core.<sup>9,43</sup> Based on the pattern, it is postulated that the mycobacterial topoisomerase I wraps around the DNA duplex.

### DNA relaxation mechanism of mycobacterial topoisomerase I

Two models have been proposed to explain the DNA relaxation mechanism catalyzed by topoisomerase I. The "free rotation model" proposes that the scissile strand is free to swivel about the intact strand as the enzyme does not directly contact the DNA downstream to cleavage site. The model implies multiple relaxation events per

round of cleavage-religation cycle. According to the second model known as "strand passage model" (or "enzyme-bridged model"), the enzyme regulates the relaxation cycle by maintaining a strong hold on the DNA upstream and downstream to the nick. A single relaxation event is permitted per cycle of cleavage-religation reaction. Studies support the former mechanism to be operating in the case of vaccinia topoisomerase I<sup>22</sup> and strand passage model for type IA topoisomerases.<sup>9</sup> The mycobacterial topoisomerase I, interacts with the upstream, as well as downstream sequences with respect to nick (Figure 7). In addition to covalent contact with +1 site, both base-specific (+2) and phosphate contacts indicate tight grip of the enzyme downstream to the cleavage site. Yet the enzyme has another stranglehold as a number of base-specific and phosphate contacts are located at a distance upstream to the cleavage site. The results are consistent with the strand passage model wherein the enzyme holds both the sides of the nick to prevent free axial rotation about the non-cleaved strand for *M. smegmatis* enzyme function.

In summary, the protein-DNA contacts mapped provide the basis for site-specificity demonstrated by mycobacterial topoisomerase I. The present analyses illustrate novel features of a type IA topoisomerase interaction with DNA. Solution structure studies in conjunction with mutational studies would aid in further delineating the relaxation mechanism employed by *M. smegmatis* topoisomerase I.

## Experimental procedures

### Purification of topoisomerase I

Topoisomerase I was purified from wild-type *M. smegmatis* SN2 cells, as described.<sup>25,44</sup> The amount of enzyme catalyzing 50% relaxation of 300 ng of supercoiled plasmid at 37°C, 30 minutes in a reaction volume of 20  $\mu\text{l}$  is defined as 1 unit, and corresponds to 2 ng of the purified protein.

### Oligonucleotide substrates

Oligonucleotide (38 mer) containing a strong topoisomerase site (STS oligonucleotide; 5'-GAG AGGCGGTTTGCATATGGGCGCTCTTCCGCTTCT) was used as the specific substrate in all the assays. The complementary oligonucleotide (non-STs oligonucleotide; 5'-AGGAAGCGGAAGAGCGCCCAATACGCAAA CCG CCTCTC) served as the non-specific substrate. When double-stranded oligonucleotides were used, 2.5-fold molar excess of unlabeled complementary strand was annealed to the labeled strand by incubating at 95°C for five minutes followed by slow cooling to room temperature. Double-strandedness of DNA was confirmed by electrophoresing an aliquot of the reaction mix through 10% (w/v) non-denaturing polyacrylamide gel. Oligonucleotides were 5' end-labeled using bacteriophage T4 polynucleotide kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP (6000



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Ci/mmol) and purified using G-50 spin column chromatography.

### Binding and cleavage reactions

The assays for non-covalent enzyme DNA complexes and cleavage reactions were performed, as described.<sup>27</sup>

### DNase I footprinting

Reaction mixtures (20  $\mu$ l) containing 40 mM Tris-HCl (pH 8.0), 20 mM NaCl, 1 mM Mg<sup>2+</sup>, 0.5 mM Ca<sup>2+</sup> and 100 fmol of radiolabeled oligonucleotides were incubated on ice for five minutes along with topoisomerase I. The samples were thereafter treated with 5 ng of DNase I (Worthington Biochemical Corp.) for 30 seconds at 24°C. The reactions were terminated by the addition of stop buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 mM EDTA). The products of the reactions were autoradiographed following separation using 16% denaturing PAGE at 2000 V for two hours.

### Methylation protection and interference assay

Topoisomerase I (150 ng or 300 ng) was incubated with the end-labeled oligonucleotides (200,000 cpm) in binding buffer (40 mM Tris-HCl (pH 8.0), 20 mM NaCl) in 10  $\mu$ l reaction volume. After increasing the volume to 50  $\mu$ l the reaction mix was treated with 1% DMS and vortexed vigorously for 1.5 minutes. The reaction was terminated by the addition of 1.5 M sodium acetate and 1 M  $\beta$ -mercaptoethanol. Following precipitation, DNA was re-suspended in 90  $\mu$ l of H<sub>2</sub>O and 10  $\mu$ l of piperidine. The samples were thereafter incubated at 90°C for 30 minutes, vacuum-dried and suspended in 6  $\mu$ l of formamide dye. Control reactions containing radiolabeled DNA without added enzyme were processed in identical fashion. Cleavage products were analyzed following separation through a denaturing 18% polyacrylamide gel at 2000 V for two hours. For interference assay, the oligonucleotide substrates were pre-methylated by treating with 1% DMS for one minute at room temperature. Modification was stopped by the addition of 50  $\mu$ l of DMS stop buffer (1.5 M sodium acetate, 1 M  $\beta$ -mercaptoethanol). DNA was precipitated and suspended in water. Binding reactions were performed, as described in methylation protection assay. DNA corresponding to free and the bound fractions were separated by electrophoresing through a native 8% polyacrylamide gel. After autoradiography the corresponding regions were excised and eluted. The samples were purified using NAP-5 columns, vacuum dried and re-suspended in 100  $\mu$ l of binding buffer. After the cleavage by piperidine the samples were electrophoresed, as described in previous section.

### Depurination and depyrimidation reaction

Purines and the pyrimidine specific reactions were performed as described earlier.<sup>31</sup> Oligonucleotides were annealed to the complementary, unmodified strand when duplex DNA was used. Binding reactions were performed as described above. The reaction products were resolved through a denaturing 18% polyacrylamide gel.

### Ethylation interference assay

Saturated solution of ethyl-nitrosourea (EtNU) in ethanol was added to end-labeled oligonucleotides (200,000 cpm) and incubated with 100  $\mu$ l of 50 mM cacodylate buffer (pH 8.0) at 50°C for 30 minutes. DNA was ethanol precipitated, and suspended in 50  $\mu$ l of water. Binding reactions and isolation of free and bound forms of DNA were as described above. Five microliters of 1 M NaOH was added to the samples and incubated at 90°C for 30 minutes and then neutralized with HCl. DNA was precipitated and suspended in 5  $\mu$ l of formamide dye and electrophoresed through a urea 16% polyacrylamide gel. The gel was then exposed for autoradiography.

### Cu(OP)<sub>2</sub> footprinting

Footprinting reactions were performed as described earlier.<sup>35,45</sup> Briefly, topoisomerase I (100-200 ng) was incubated with 1 pmol end-labeled DNA (10<sup>5</sup> cpm) in 170  $\mu$ l of reaction buffer containing, 20 mM Tris-HCl (pH 8.0) and 20 mM NaCl for five minutes on ice. The reaction was initiated by the addition of 4 mM 1,10-phenanthroline, 0.3 mM CuSO<sub>4</sub> and 58 mM 3-mercaptopropionic acid and quenched after one minute at 25°C, by the addition of 5  $\mu$ l of 100 mM 2,9-dimethyl-1,10-phenanthroline. Following phenol-chloroform extractions DNA was precipitated, purified and the products were electrophoresed through a denaturing 18% polyacrylamide gel.

### KMnO<sub>4</sub> footprinting

Preformed DNA-protein complex was incubated with 2 mM KMnO<sub>4</sub> for one minute at 25°C.<sup>46</sup> The reaction was stopped with 40 mM  $\beta$ -mercaptoethanol and 0.3 M sodium acetate. Following ethanol precipitation and piperidine cleavage, the reaction products were electrophoresed through a denaturing 18% polyacrylamide gel.

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