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DNA topoisomerases from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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Abstract

DNA topology has a major influence on vital processes such as replication and transcription in all organisms. DNA topoisomerases catalyze the topological reactions and are essential for cell survival. We have been working on different classes of DNA topoisomerases from mycobacteria to understand their regulation, crucial role in important cellular functions and in expression of virulence genes. The studies are also aimed at developing new therapeutics against tuberculosis, which has reemerged as the number one killer worldwide. Molecular cloning and overexpression of the genes for DNA gyrase (a type II topoisomerase found only in bacteria) from mycobacteria in our laboratory has paved the way for detailed characterization of the enzyme. In parallel, DNA topoisomerase I from mycobacteria is also characterized. The studies have unraveled the unique features of these enzymes from mycobateria.

Keywords: Mycobacteria, topoisomerases, DNA gyrase, supercoiling.

1. Introduction

Although the information content of DNA is independent of its topology, yet it provides an evolved means of regulation of many vital cellular processes. In most of the organisms, genome is negatively supercoiled as their underwound state represents higher intrinsic free energy which can be utilized to open up the DNA duplex. The intricate relationship of DNA topology and its influence on cellular processes involving DNA transactions has been the subject of research over the past few decades. Considering its importance in transcription, replication and recombination, it is not surprising that the cell possesses control mechanisms to monitor and modulate the global superhelicity of the DNA [1]. The topological changes accompanying these DNA transaction processes are catalyzed by a group of enzymes called the topoisomerases [2, 3].

Topoisomerases are classified into two groups, type I and type II [4]. Type I topoisomerases make a single-stranded break into the DNA, pass the other strand of DNA through the nick and then reseal it, changing the linking number by steps of one. Type I enzymes can be further classified into two subclasses–type IA and type IB. Type IA enzymes, repre-

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sented by the prokaryotic proteins, cleave the single-stranded DNA forming a 5′-phosphotyrosine covalent intermediate. Contrastingly, type IB enzymes make a 3′ phosphotyrosine covalent intermediates during the DNA relaxation reaction. Vaccinia virus and human topoisomerase I are the examples of the latter subclass. DNA gyrase topoisomerase IV and eukaryotic topoisomerase II belong to type II group of enzymes, which create transient double strand breaks into one segment of DNA and pass an intact duplex through the broken duplex before religation of the phosphodiester bond. Thus, topological interconversions catalyzed by class II enzymes result in alteration of linking number in steps of two.

Amongst all topoisomerases, DNA gyrase enjoys a special status, as it is the only known enzyme catalyzing negative supercoiling reaction and hence obvious drug target. DNA gyrase has been isolated from many bacterial species, and best characterized from *Escherichia coli* [5]. It consists of two subunits that form an A_2B_2 heterotetrameric complex, constituting the active form of the enzyme. DNA gyrase is the only enzyme that can introduce negative supercoiling into DNA in an ATP-dependent reaction. It also relaxes negatively supercoiled DNA in the absence of ATP. Apart from these reactions, DNA gyrase is shown to perform a number of topological interconversions of DNA molecule such as knotting/unknotting and catenation/decatenation [6]. The gyrase A protein (GyrA) determines sensitivity to the quinolone drugs and contains the DNA cleavage/reunion activity [7]. Gyrase B protein (GyrB) harbours an intrinsic ATPase activity which is sensitive to novobiocin, coumermycin and other coumarin drugs [5]. The second bacterial type II enzyme, topoisomerase IV, has strong decatenase activity and seems to be essential for chromosome segregation [8].

The prokaryotic topoisomerase I has been first characterized from *E. coli* [9]. Topoisomerase I has also been isolated from a few other bacterial sources such as *Micrococcus luteus* [10] and *Fervidobacterium islandicum* [11]. Prokaryotic type I topoisomerases catalyze the relaxation of negatively supercoiled DNA in an energy-independent manner. Other reactions catalyzed by these enzymes include knotting and catenation of single-stranded DNA or duplex DNA having a nick in one of the strands [12]. Moreover, the enzyme plays a critical role in the maintenance of *in vivo* superhelical density of the genome in conjunction with the supercoiling activity of DNA gyrase. Topoisomerase I has also been implicated to have a role in DNA replication, transcription and recombination events [13].

The importance of DNA supercoiling in virulence gene expression is the focus of study in several laboratories. In addition to the influence on DNA replication, transcription and other vital processes, the topoisomerases seem to influence virulence gene expression in many pathogenic bacteria [14]. The expression of many virulent genes has been shown to be influenced by factors that modulate supercoiling as exemplified by the regulation of invasion genes of *Shigella flexneri* and enteroinvasive *E. coli* [15]. The site-specific recombinase regulating the expression of type 1 fimbriae in *E. coli*, needed for the colonization of the host, was also found to be sensitive to supercoiling [16].

The genus *Mycobacterium* is of immense public health importance as *Mycobacterium tuberculosis*, *Mycobacterium leprae* and many other pathogenic species belong to this group. Tuberculosis continues to rage serious health concerns in all the developing countries. Reappearance of tuberculosis in the western world and emergence of multiple drug-resistant

Table I
Antibacterial drugs for tuberculosis chemotherapy

Drug	Functions affected	Target molecules	Molecular basis resistance
Streptomycin	Prokaryotic protein translation	S12 ribosomal protein and 16S rRNA	Point mutation in rpsL and rrs locus
Rifampicin	Elongation of full length transcripts	β-subunit of RNA polymerase	Mutations in rpoB gene
Floroquinolones	Supercoiling	DNA gyrase	gyrA
Isoniazid and ethionamide	Fatty acid elongation and mycolic acid biosynthesis	Enoyl reductase and catalyase peroxidase	Mutation in *Kat*G, *inh*A
Ethambutol	Arabinogalactan biosynthesis	Arabinosyl transferase	–
Pyrazinamide	Change in the pH	Amidase	Mutations in *pnc*A

clinical strains of *M. tuberculosis* in the last several years have raised a global concern. The link between dramatic surge in tuberculosis and HIV infections is well established. Both primary and acquired resistance against the broadspectrum antibiotics streptomycin, rifampicin, fluoroquinolones has been reported (Table I). Development of resistance to other major antimycobacterials such as ethambutol, isoniazid and pyrazinamide has further compounded the problem (Table I).

Due to inherent complexity, mycobacterial biology and immunopathology are not well understood to the extent efforts have been made in this direction. Thus, in addition to exploring topoisomerases as molecular targets, detailed characterization of topoisomerases from mycobacteria is also important to understand the basic biology of the organism. A better understanding of the macromolecular events that regulate the physiology of the organism would facilitate the design of improved chemotherapeutic regimens for preventing as well as managing mycobacterial infections. The slow growth rate of mycobacteria and their survival in host monocytes and macrophages is suggestive of some unusual regulatory features of DNA transaction events, which in turn could be linked to the DNA modulatory action of topoisomerases. Our major emphasis thus is to study the DNA topoisomerases from mycobacteria aimed at developing them as drug targets and also to explore their role in vital cellular processes and expression of virulence genes. In the following section we present and discuss some of the research highlights.

2. DNA gyrase from *M. smegmatis* and *M. tuberculosis*

DNA gyrase is unique amongst all topoisomerases, as it is present only in prokaryotes with extraordinary clinical importance. Since the enzyme is a proven molecular target, our initial efforts were focused on characterization of DNA gyrase from mycobacteria. We reported the enzyme activity from *M. smegmatis* in 1980 soon after its characterization from *E. coli* [17]. Cloning the genes for DNA gyrase was preferred over the isolation of enzyme by growing large amounts of bacteria considering the complexities in dealing with mycobacterial cultures. We used several strategies in our attempts to clone *gyrA* and *gyrB* genes of *M. smegmatis* and *M. tuberculosis*. One of the first strategies relied upon functional complementation of *E. coli* strains having *gyrA* or *gyrB* temperature-sensitive phenotypes. This ap-

proach proved to be unsuccessful. A parallel attempt was based on PCR method using degenerate primers designed from conserved regions after multiple alignment of known gyrase sequences. A third alternative was to clone the genes based on sequence homology using southern hybridization technique. All the three approaches turned out to be unsuccessful. The possible reasons for failure in these efforts could be due to the difference in promoter elements and regulation of transcription, lack of sufficient homology, difference in G + C content and codon usage.

Genes from *M. tuberculosis* DNA gyrase were eventually cloned by southern hybridization using *S. sphaeroodes gyrB* gene [18] as probe [19, 20]. Using cloned *gyrA* and *gyrB* DNA fragments from *M. tuberculosis*, *M. smegmatis gyrA* and *gyrB* were readily cloned [21]. Sequencing of the genes provided unexpected results with respect to gene organization and structure. The genes, *gyrA* and *gyrB*, were next to each other in both the mycobacterial species in contrast to *E. coli* where *gyr*B and *gyr*A are located far apart at 83.5 and 48 min, respectively (Fig. 1). Since actinomycetes are considered borderline eukaryotes, the operon-like organization of *gyrB* and *gyrA* in mycobacteria is rather surprising. The functioning of *gyr*B-*gyr*A as a dicistronic unit in *M. smegmatis* has been demonstrated by us previously [22]. The single promoter located upstream of *gyr*B is characterized and shown to be specific to mycobacteria. Surprisingly, unlike *M. smegmatis,* multiple promoters are involved in the transcription of *gyrBA* operon in *M. tuberculosis* [23].

Apart from identical gene organization, the genes from the two species of mycobacteria are highly homologous. The pairwise comparisons of the two proteins showed that the GyrA of *M. smegmatis* shares 88.5% identity and 93.9% similarity with that of *M. tuberculosis* at amino acid level. *M. smegmatis* GyrB is also highly homologous (86% amino acid sequence identity) to *M. tuberculosis* GyrB protein [24]. A distinctive feature of mycobacterial GyrB is the absence of 165 amino acid contiguous stretch found towards the carboxy

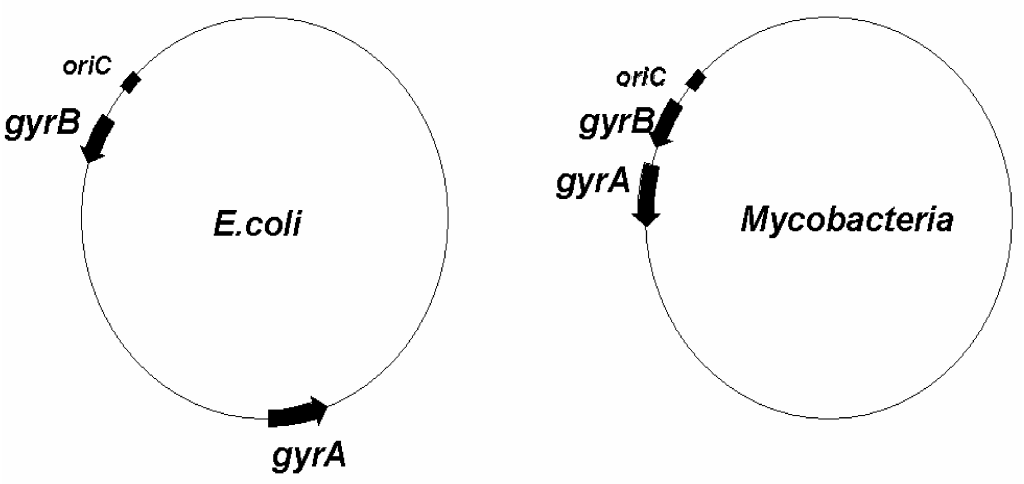

FIG. 1. Organization of *gyr* genes in the genomes of *E. coli* and mycobacteria. Unlike in *E. coli* where the two genes are far apart, the genes are next to each other in both *M. smegmatis* and *M. tuberculosis* located close to ori. They are also organized as a dicistronic unit.

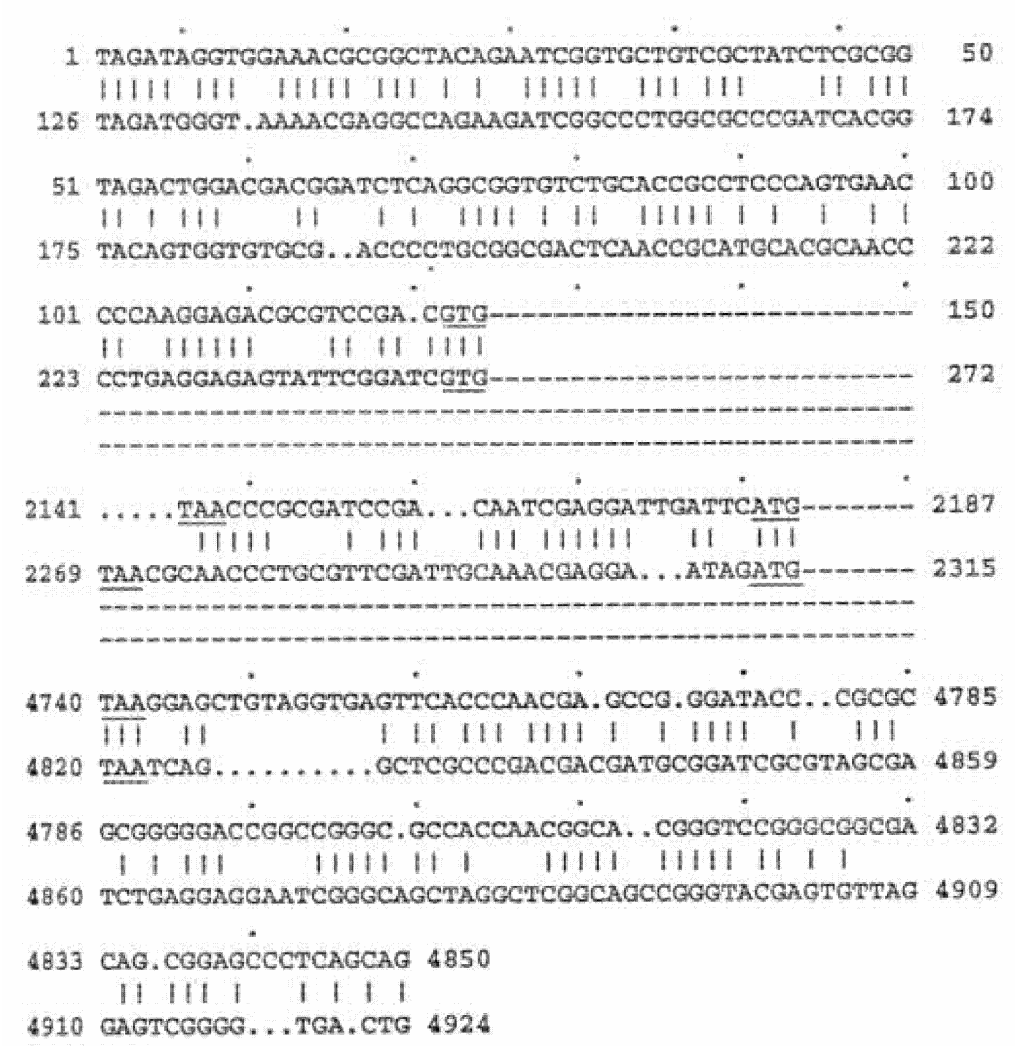

FIG. 2. Alignment of flanking sequences in *gyr* locus. The regions of *M. tuberculosis* and *M. smegmatis* gyr locus were analyzed using GAP (UWGCG). Dots represent the reading frames, while the vertical bars indicate the identical nucleotide residues in both the species. The start and stop codons are underlined.

terminus of *E. coli* GyrB. The region appeared to be auxillary, as important domains for gyrase structure and function are well conserved in all the bacterial species. The importance (if any) of this additional stretch of amino acids for *E. coli* gyrase was addressed by a series of experiments [25]. The nucleotide sequences in the flanking regions of the *gyr*B and *gyr*A genes from the two species also show considerable sequence similarity (Fig. 2). Moreover, the order of genes in the region, *dna*A, *dna*N, *rec*F, *gyr*B and *gyr*A, seems to be conserved in identical fashion in both the species. These data strengthen the allelic nature of the genes and identical location on the chromosome and indicate the evolution from a common ances-

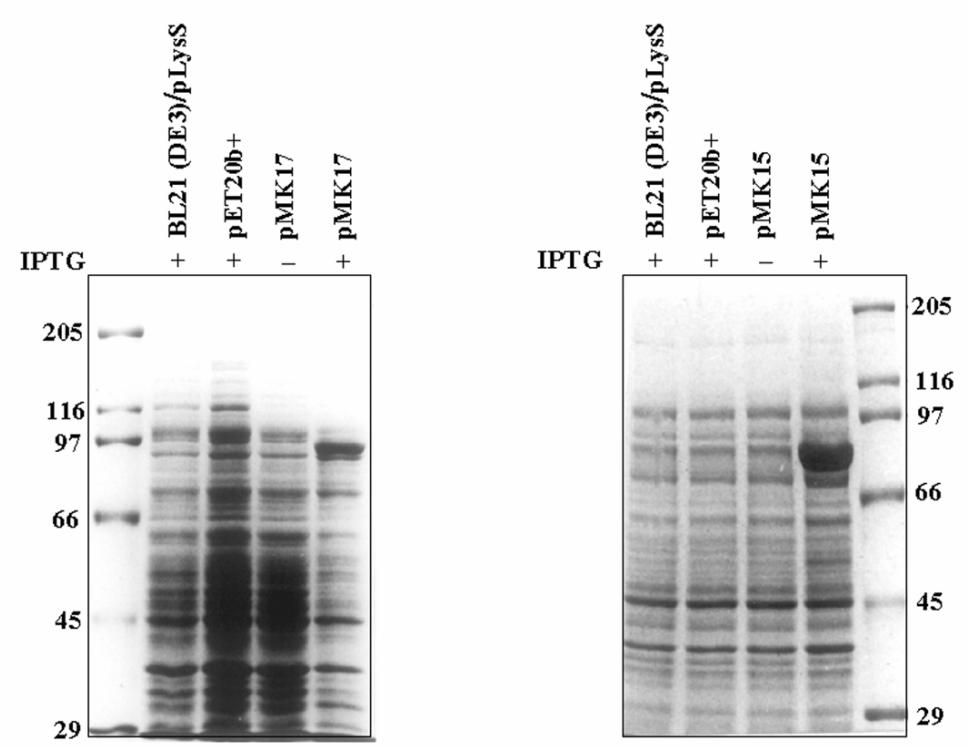

FIG. 3. Expression profile of *M. tuberculosis gyr*A and *gyr*B: *M. tuberculosis gyr*A and *gyr*B genes were cloned into pET20b+plasmid (pMK17 and pMK15, respectively) and overexpressed in *E. coli* BL21 (DE3)/pLysS using standard gene manipulation techniques described earlier [24]. The log phase cultures were induced with 0.3 mM isopropylthio-*β*-D-galactoside (IPTG) and further processed as described previously (24). The bacteria were lysed in sample buffer and analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The IPTG addition (induction) is represented by '+'. Protein molecular weight markers are shown in right most lane and sizes are indicated.

tor. Taken together the above findings suggest that the genes from *M. smegmatis* DNA gyrase could be used to carry out genetic studies to address the *in vivo* role of the enzyme.

As a prelude for structure–function studies, *gyr*A and *gyr*B genes from both the species of mycobacteria have been engineered for hyperexpression in suitable expression vectors. The strategy for hyperexpression of *gyr*B and *gyr*A from *M. smegmatis* has been previously described [21]. In a somewhat similar approach, *gyr*B and *gyr*A of *M. tuberculosis* have been engineered for overexpression. A representative expression profile is depicted in Fig. 3. The overexpression of genes has provided the basis to develop strategies to screen new inhibitors for DNA gyrase. In addition, it has also facilitated the structure–function analysis and biochemical studies on mycobacterial enzymes [26].

3. Topoisomerase I from *M. smegmatis* and *M. tuberculosis*

We had to employ altogether a different approach to characterize topoisomerase I. Initially, we resorted to functional complementation approach to clone the genes for topoisomerase I

from *M. tuberculosis* and *M. smegmatis*. For this purpose, genomic libraries of *M. smegmatis* and *M. tuberculosis* were constructed in both pUC19 and pBR322 and then transformed into an *E. coli* strain (AS17) which is temperature sensitive for topoisomerase I production [27]. These rescue experiments were not successful. Unusual regulatory features, difference in control elements and codon usage could be some of the reasons for these results. An alternative approach was to investigate the biochemical properties of the enzyme isolated from mycobacterial cells. Topoisomerase I was purified to homogeneity from *Mycobacterium smegmatis*. The purified 110 kDa protein catalyses DNA relaxation reaction of negatively supercoiled DNA in a Mg^{++}-dependent manner [28]. The mycobacterial topoisomerase I is similar to type IA topoisomerases in many properties including catenation and knotting of single-stranded DNA. The enzyme introduces single-stranded nicks and the reaction proceeds through an intermediate involving a 5′-phospho-tyrosine covalent protein-DNA adduct. In spite of these similarities with respect to biochemical properties of type IA topoisomerases, *M. smegmatis* enzyme shows some distinctive features when compared to the prototype of *E. coli* topoisomerase I. (a) The enzyme is relatively stable at higher temperatures. The mycobacterial enzyme retains the relaxation activity at 55°C, while the *E. coli* enzyme loses its activity above 42°C indicating that the *M. smegmatis* topoisomerase I is a more thermo-stable enzyme. (b) Unlike the *E. coli* enzyme, it is not inhibited by spermidine, an intracellular polyamine. (c) The enzyme has broader pH optima as compared to the *E. coli* enzyme. (d) The alignment of *E. coli* and *M. tuberculosis* topoisomerase I sequences demonstrates the absence of Zn-finger motifs in the mycobacterial enzyme (Fig. 4). The cysteine residues present in *M. smegmatis* enzyme do not coordinate Zn^{++} and on their modification, the enzyme retains the activity. These results suggest the presence of a different DNA-binding motif unlike that in *E. coli* [28, 29] and *Bacillus subtilis* [30] topoisomerase I, where the Zn-finger motif has been implicated in DNA binding. The absence of a 'typical' DNA-binding motif characteristic of other prokaryotic topoisomerases led us to look into the DNA-binding properties of the enzyme before identifying the DNA-binding motif. A major question asked is whether the enzyme recognizes specific sequences in the DNA although the other prokaryotic topoisomerases do not exhibit high degree of sequence specificity [31]. Experiments in this regard led us to identify recognition sequences in pUC19 sequence. A unique feature of the enzyme seems to be the ability to recognize both single- and double-strand DNA with high affinity [31]. Subsequently, a large number of strong topoisomerase sites have been mapped on genomic DNA fragments of *M. smegmatis*

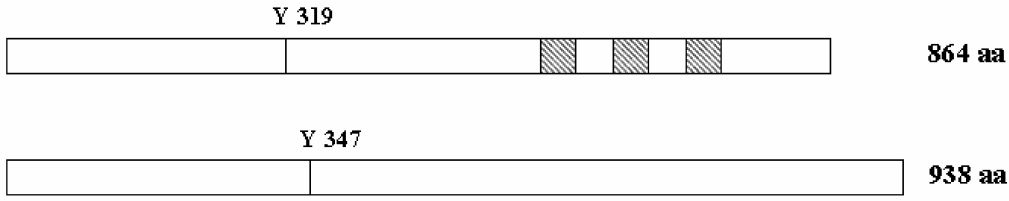

FIG. 4. Schematic alignment of topoisomerase 1 from *E. coli* and *M. tuberculosis*: The primary sequence of *M. tuberculosis* topoisomerase 1 (938 amino acids) was aligned with the topoisomerase 1 of *E. coli* (864 amino acids). Conserved active site tyrosine is indicated. The cysteine clusters present in *E. coli* topoisomerase 1 are depicted by open boxes. Note the absence of this cluster in *M. tuberculosis* enzyme.

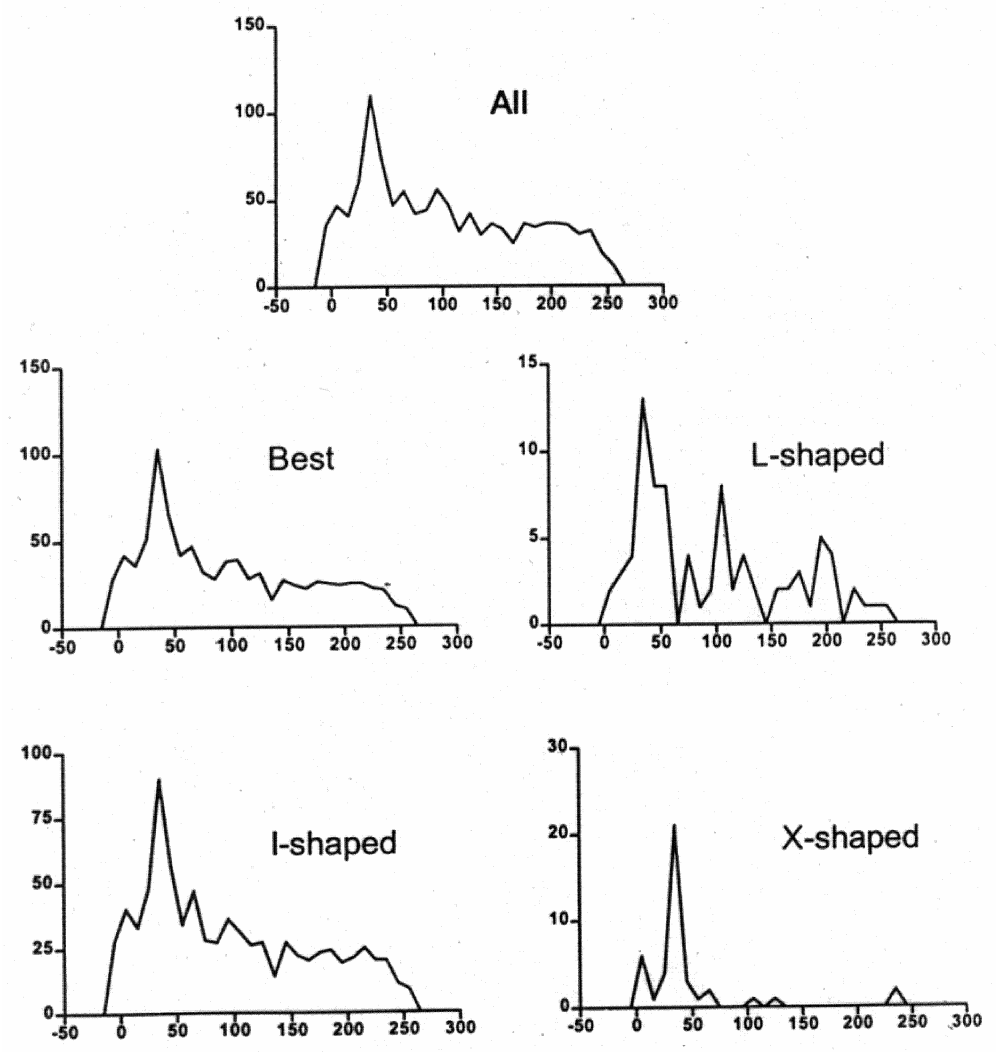

FIG. 5. The distribution of different kinds of intrinsic transcription terminators in *M. tuberculosis* genome. The genome sequence is subjected to analysis using GeSTer as described in Unniraman *et al*. [34, 35]. The numbers in the X axis represent the nucleotide distance from the translational stop codon (0) and those in Y axis the number of terminators.

and *M. tuberculosis* by designing a new technique [32]. The outcome of these experiments is the design of consensus sequence to address the site-specific interaction of the topoisomerase I with DNA. The molecular contacts involved in the site-specific recognition were mapped by a variety of footprinting techniques to further delineate the specific interactions [33].

In parallel, the regulation of expression of topoisomerases was undertaken which would, in turn, provide a handle to study virulence gene expression. These studies led to the eluci-

dation of mycobacterial promoters specific to mycobacteria, relaxation-stimulated transcription in mycobacteria, elucidation of promoter consensus sequences [22, 23]. In addition, different classes of intrinsic transcription terminators were identified which were shown to be functional in transcription termination both *in vitro* and *in vivo*. Figure 5 depicts the distribution of different kinds of intrinsic terminators in *M. tuberculosis*. The results of such an analysis blending bioinformatics, genomics and molecular biology techniques reveal that the mechanism of intrinsic transcription termination is conserved in eubacteria. However, alternative strategies exist to pause, arrest and release of the RNA polymerase from the elongation complex [34, 35]. Results from our laboratory over the last few years indicate that transcription elongation complex also contains DNA gyrase subunits [Richa Gupta *et al*., unpublished]. Such an association of RNA polymerase with DNA gyrase appears to be maintained throughout the different stages of growth in *M. smegmatis*. This intimate association of the two important components of cellular machinery may have important bearing with respect to RNA polymerase assembly, promoter recognition, open complex formation, processive elongation, etc. Current experiments are attempting to delineate the functional basis of this interaction. At present, no information is available on mycobacterial topoisomerase I gene promoter structure, regulation and interaction with other proteins. Understanding these features would reveal a global picture of the role of topoisomerases in influencing other important processes in mycobacteria.

In conclusion, our research with mycobacterial topoisomerases has generated a wealth of information on this important class of enzymes (36). Unusual features of the enzymes form the basis to develop them as molecular targets. One such effort has led us to develop a new class of gyrase inhibitors specific for mycobacterial gyrase with mechanism of action distinct from quinolones and coumarin class of compounds (37). Studies on transcriptional regulation and termination have led to some important new observations on the biology of eubacteria (38). The information obtained and the tools generated so far would go a long way in unraveling the integral role of topoisomerases in major molecular events of formidable mycobacteria.

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