

Regulation of DNA gyrase operon in *Mycobacterium smegmatis*: a distinct mechanism of relaxation stimulated transcription

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Abstract

Background: The topological state of DNA is a result of the diverse influences of various topoisomerases present in the cell. Amongst these, DNA gyrase is the only enzyme that is capable of supercoiling DNA. In all the eubacterial cells tested so far, DNA gyrase has proved to be essential for survival. We have earlier cloned *gyr* genes from *Mycobacterium smegmatis*. Unlike the situation in *Escherichia coli*, genes encoding the two subunits of gyrase are present as a contiguous stretch in the *M. smegmatis* genome.

Results: We have demonstrated that the two subunits are encoded by a single dicistronic message, with the transcriptional start site mapping 57 base pairs upstream of the putative translational start of the *gyrB* ORF. The *gyr* promoter is specific to *M. smegmatis* and does not function in *E. coli*. We have

shown that the synthesis of DNA gyrase in *M. smegmatis* is induced by novobiocin—a known inhibitor of gyrase. Short fragments encompassing the promoter region, when cloned in a promoter selection vector, do not show any response to changes in supercoil levels. Larger fragments show a supercoil sensitive behaviour, as seen in the genomic context.

Conclusions: The gene structure and the transcriptional organization of the *gyr* operon suggest an overall regulatory scheme that is unique to mycobacteria. In contrast to *E. coli*, promoter and regions in its vicinity are not sufficient to confer supercoil sensitivity. Promoter distal regions—600 bp downstream of the promoter—appear to be necessary for relaxation-stimulated transcription in *M. smegmatis*.

Introduction

DNA in eubacterial cells is negatively supercoiled. The level of supercoiling of the genome is known to influence practically all processes that involve DNA as a substrate—replication, transcription and recombination (Wang 1985, 1996 and references therein). Supercoiling is known to correlate with an increased rate of transcription initiation and a greater number of transcribing complexes; consistent with the observation that binding of the RNA polymerase leads to an unwinding of the double helix in the open complex. Thus, the negative supercoiling stimulates transcription in general. The net level of DNA supercoiling in the cell is a result of the opposing influences of the

relaxation brought about by topoisomerases I and IV on the one hand and the supercoiling activity of DNA gyrase on the other (Pruss *et al.* 1982; Richardson *et al.* 1984; Zechiedrich *et al.* 1997).

DNA gyrase is a heterotetrameric protein with two types of subunits—GyrA and GyrB. It is the only enzyme which is capable of introducing negative supercoils into DNA. The genes encoding the two subunits are widely separated in the *Escherichia coli* genome (Bachmann 1987). Since DNA gyrase is involved in increasing the supercoiling of DNA, it is not surprising that the expression of the *gyr* genes is induced by the relaxation of the DNA template (Menzel & Gellert 1983). Relaxation stimulated transcription (RST) provides a convenient strategy for the cell to homeostatically maintain the level of supercoiling.

An analysis of the *gyrA* and *gyrB* loci of *E. coli* has shown that the sensitivity of the *gyr* genes to

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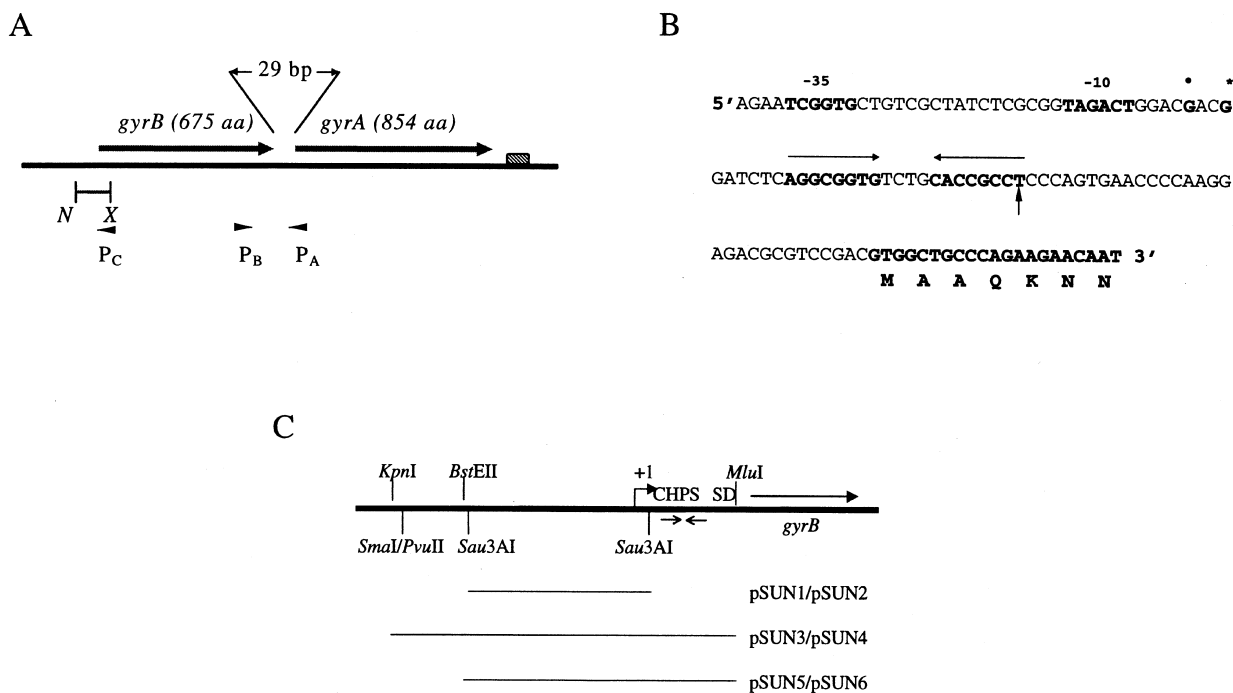


Figure 1 (A) Organization of *gyr* genes in *M. smegmatis*. N–X represents the *Nru*I–*Xho*I fragment used for S1 nuclease analysis. The position of various primers discussed in the text is indicated by arrowheads. The filled box indicates the position of the putative transcription terminators; (B) Promoter elements upstream of the start site. The start site mapped by primer extension (*) and by S1 nuclease analysis (●) are shown. The CHPS (→ ←) is shown along with the primer extension products mapping near it (†); (C) Partial restriction map of the region around the promoter along with short fragments cloned into pSD7. The position of the CHPS is shown as → ←.

supercoiling is an intrinsic property of DNA elements in and around the promoter, particularly the –10 region itself (Menzel & Gellert 1987a,b; Straney *et al.* 1994). Carty & Menzel (1990) have also implied a role for a *trans* factor(s) in RST, which could be titrated out *in vitro*. Despite such analyses, a clear molecular mechanism for RST has not emerged. In addition, the *gyr* genes are certainly not the only genes which exhibit RST. Many other promoters have also been shown to respond similarly to changes in supercoiling (Jovanovich & Lebowitz 1987; Urios *et al.* 1990; O'Byrne *et al.* 1992; Kohno *et al.* 1994; Wu & Dutta 1995).

The present work describes our analysis of *gyr* genes from *Mycobacterium smegmatis*—their organization and regulation. *M. smegmatis* is a fast growing nonpathogenic member of the genus *Mycobacterium*. It has been used as a model system for analysing the molecular biology and biochemistry in mycobacteria for over three decades (McFadden 1990). Earlier, we have cloned the genes encoding DNA gyrase from *M. smegmatis* (Madhusudan & Nagaraja 1995). Here we show that the two genes are part of a single operon, with

the transcription start site located upstream of the *gyrB* gene. The *gyr* promoter functions only in mycobacteria and not in *E. coli*. The *gyr* genes are induced when the cells are treated by novobiocin—a known inhibitor of DNA gyrase. Our results imply, unlike the situation in *E. coli*, that promoter distal regions are essential for RST in *M. smegmatis*.

Results

Organization of the *gyr* genes in *M. smegmatis*

In contrast to the organization in *E. coli*, the *gyrA* and *gyrB* genes are present on a single contiguous stretch in *M. smegmatis* (Fig. 1A). The *gyrB* gene is present upstream of *gyrA*, with a short intergenic region of 29 nucleotides (Madhusudan & Nagaraja 1995). An analysis of the sequence in and around the *gyr* genes showed the presence of putative promoter elements upstream of *gyrB* and strong rho-independent transcription terminator-like sequences downstream of *gyrA*. The short intergenic region was devoid of promoter or

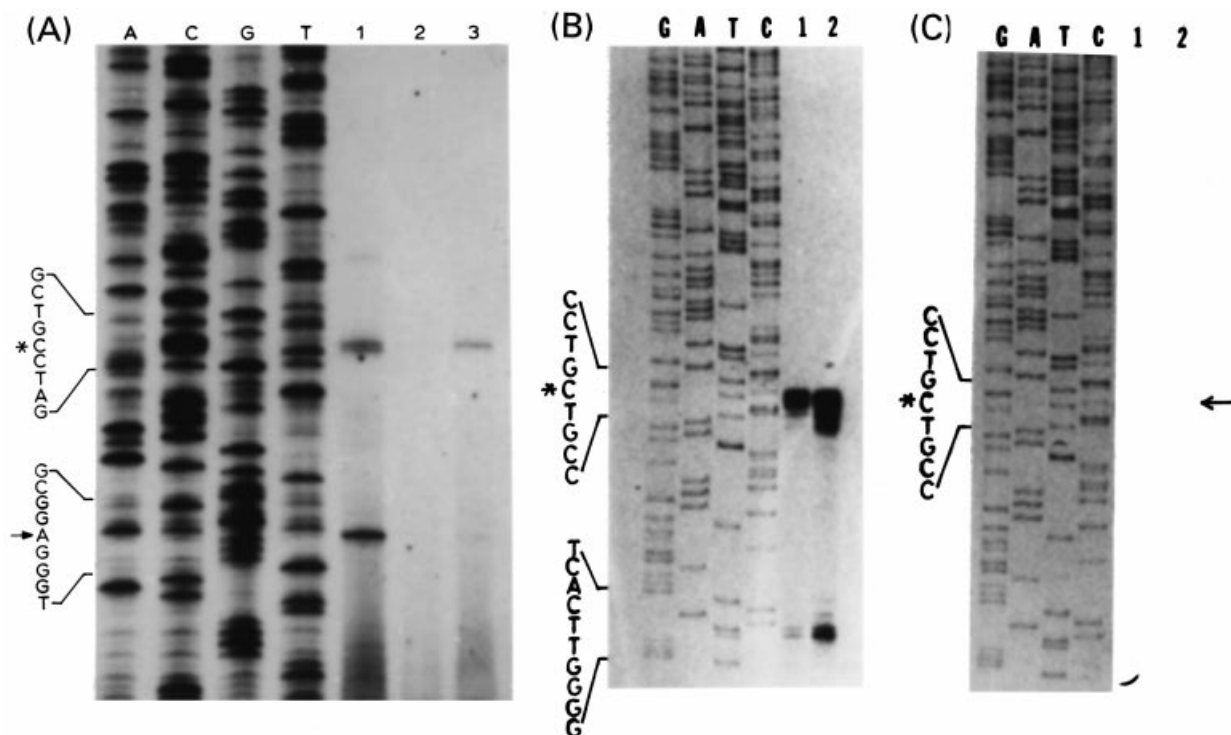


Figure 2 Identification of the transcription start site. (A) Primer extension: Lane 1, Reaction at 42 °C after denaturation at 70 °C; lanes 2 and 3, at 50 °C after denaturation at 95 °C. Lanes 1 and 3, reaction with RNA isolated from cells treated with novobiocin; lane 2, from untreated cells. All reactions were performed with 10 µg of RNA and 10 pmoles of end-labelled primer P_C and 200 U of Superscript II reverse transcriptase (B and C) S1 nuclease analysis. 300 mg of RNA was co-precipitated and denatured with 5' end labelled *Nml-XhoI* fragment shown in Fig. 1A. After hybridization at 65 °C for 16–20 h the samples were treated with 400 U (lane 1) and 200 U (lane 2) of S1 nuclease for 45 min at 37 °C. (B) Hybridization by rapid cooling to 65 °C. (C) Hybridization by slow cooling to 65 °C. The dideoxy-nucleotide sequencing ladders (G, A, T and C) are shown.

terminator-like sequences, thus suggesting a dicistronic arrangement. A RT-PCR analysis of *M. smegmatis* RNA with primers (Fig. 1A) specific to *gyrA* (P_A) and *gyrB* (P_B) showed an amplification of a 470-bp product encompassing the intergenic region, proving that the two genes are part of a single dicistronic message. Primer extension analyses only showed the presence of a start site upstream of *gyrB*, in agreement with the above conclusion (see next section).

Mapping the transcription start site of the *gyr* mRNA

To identify the promoter elements upstream of *gyrB*, we determined the 5' start site of the *gyr* mRNA by both primer extension and S1 nuclease analysis. Initially, when primer extension was carried out at 42 °C, two products were generated (Fig. 2A, lane 1), which could be attributed to the following reasons: (i) presence of two promoters, (ii) processing of the full-length

message, or (iii) some local secondary structure in the RNA. The third possibility seems most likely since only the longer product was generated when the extension was performed at 50 °C (Fig. 2A, lane 3). Under these conditions, fewer intramolecular structures would be formed. These experiments were carried out with RNA isolated from cells treated with novobiocin. Novobiocin is a known inhibitor of gyrase (Gellert *et al.* 1976) and, by virtue of the inhibition, causes relaxation of the DNA inside the cell (Drlica & Snyder 1978). In turn, relaxation has been shown to induce gyrase expression in *E. coli* (Menzel & Gellert 1983). We find a similar induction of transcription in *M. smegmatis* cells (Fig. 2A, compare lanes 2 and 3). The reactions in all three lanes were carried out with equal amounts of RNA. Similar results were obtained by S1 nuclease analysis, although the +1 site mapped a few bases upstream with respect to that obtained by primer extension (Fig. 2B,C). This is not surprising considering the heterogeneity in cleavage at the

Table 1 Promoter activity in *E. coli* and *M. smegmatis*

Plasmid	<i>E. coli</i>			<i>M. smegmatis</i>		
	c.f.u. ($\times 10^6$)		CAT activity	c.f.u. ($\times 10^6$)		CAT activity
	Kn ^r	Kn ^r Cm ^r		Kn ^r	Kn ^r Cm ^r	
pSD7	3.2	0	0.2 ± 0.1	5.1	0	0.3 ± 0.1
pSUN1	3.5	0	0.3 ± 0.1	4.0	4.1	7.0 ± 0.4
pSUN2	2.4	0	0.1 ± 0.1	5.8	0	0.7 ± 0.2
pSUN3	3.0	0	0.2 ± 0.1	6.3	6.2	24.7 ± 1.2
pSUN4	2.3	0	0.3 ± 0.1	6.1	0	0.7 ± 0.2
pSUN5	3.0	0	0.2 ± 0.1	5.5	5.3	21.4 ± 1.1
pSUN6	3.6	0	0.1 ± 0.1	5.2	0	0.9 ± 0.2

CAT specific activity was calculated as described in Experimental procedures.

junction between the RNA–DNA duplex and single-stranded DNA by S1 nuclease (Berk 1989). The putative –10 and –35 regions upstream of the transcription start site are shown in Fig. 1B. Interestingly, a palindromic sequence (cruciform/hairpin potential sequence, CHPS) is located 10 nucleotides downstream of the transcription start site (Fig. 1B). Such sequences could adopt a hairpin-like structure in mRNA and/or extrude out as a cruciform DNA in a supercoil-dependent manner (Lilley 1985). Such a structure would explain the second extension product or protected fragment that was detected in the above experiments. The theoretical estimate for the free energy of melting of the hairpin is –17.30 kcal/mol and therefore once formed, the structure would be extremely stable. A similar primer extension analysis with P_A failed to detect any *gyrA*-specific transcriptional start site, again implying that transcription is

driven from the solitary promoter located upstream of *gyrB*.

Promoter activity in *E. coli* and *M. smegmatis*

Short fragments encompassing the putative promoter (Fig. 1C) were cloned in the promoter selection shuttle vector pSD7 (Dasgupta *et al.* 1993) at the unique *Bam*HI site present upstream of a promoterless CAT gene. If the cloned fragment contains a functional promoter in the correct orientation, the CAT gene would be expressed and would confer chloramphenicol resistance to the cell. Earlier workers have shown that the CAT-specific activity in such constructs reflects the relative strength of the promoter (Gorman *et al.* 1982). Both *E. coli* and *M. smegmatis* cells transformed with these constructs were tested for resistance to kanamycin (Kn^r) and chloramphenicol (Cm^r) (Table 1). None of the *E. coli* transformants was resistant to chloramphenicol, while the constructs with the promoter in the correct orientation conferred resistance to *M. smegmatis* cells. CAT specific activity in crude cell extracts essentially paralleled the result (Table 1), indicating that the promoter is specific to mycobacterium and does not function in *E. coli*. These findings are supported by the observation that the *M. smegmatis* genes did not complement the *E. coli gyrA* and *gyrB* temperature sensitive strains when present under their native promoter. On the other hand, when the *M. smegmatis gyrA* was expressed under P_{trc}, and *gyrB* under P_{lac}, they rescued the respective *E. coli* mutants. Interestingly, the cells harbouring pSUN3 and pSUN5 show 3- to 3.5-fold higher CAT activity than those harbouring pSUN1 when the cells were grown in LB medium, and the difference increased to six to sevenfold in YK medium. There was a further increase

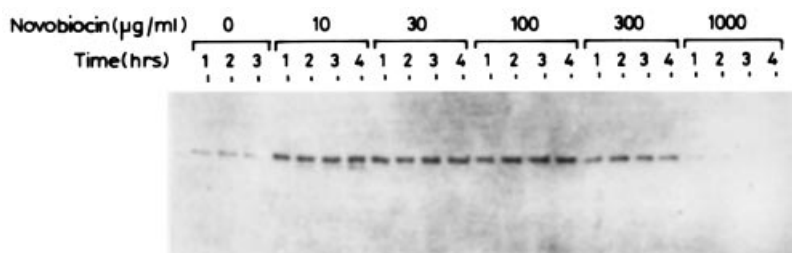


Figure 3 Immunoblot analysis of protein extracts from *M. smegmatis*. Cells were grown to mid-log phase, split into 5 mL aliquots and treated with different amounts of novobiocin. Cells were harvested at different times and frozen at –70 °C. Prior to the immunoblot, the cells were thawed, washed and sonicated. The protein extracts were quantified and 6 µg of protein extract was loaded on each lane. The protein was transferred on to a PVDF membrane, probed with anti-GyrA polyclonal antibodies and developed with anti-rabbit IgG–HRP conjugate using hydrogen peroxide and TMB.

(40-fold) when the cells were grown in Starvation Medium (YK medium with 0.5% glycerol instead of 2%). It should be pointed out that pSUN1 lacks the cruciform/hairpin potential sequence (CHPS) and putative Shine–Dalgarno (SD) sequence which are present in the other two constructs (Fig. 1C). Both these elements have been shown to stabilize the downstream mRNA against degradation in *E. coli* (Hansen *et al.* 1994; Agaisse & Lereclus 1996).

Analysis of RST at the genomic level

To establish RST in *M. smegmatis*, we assessed changes in steady-state levels of the GyrA protein. Immunoblot analysis using polyclonal antibodies raised against *M. tuberculosis* GyrA protein showed that DNA gyrase was induced threefold on treatment with novobiocin (Fig. 3). The response of cells reached a maximum within 4–5 h of treatment with the drug. The response of cells appears to be biphasic with respect to drug concentrations. At low to intermediate concentrations of the drug (10–300 $\mu\text{g}/\text{mL}$), there is an increase in the steady-state level of GyrA. At higher concentrations (1 mg/mL), the operon appears to be refractory to the drug, probably because the cells die even before they mount an adaptive response. Thus, we see that treatment with novobiocin leads to an increase in the *gyr* message (Fig. 2A, compare lanes 2 and 3) and this leads to an increase in steady state levels of the *gyr* proteins.

Role of promoter proximal regions in RST

The presence of a cruciform potential sequence near the 5' end of the *gyr* message suggests a possible mechanism for RST in *M. smegmatis*. Cruciforms in DNA have been shown to extrude in a supercoil dependent manner (Lilley 1985). In addition, cruciform structures overlapping promoter elements or within the gene can down-regulate gene expression (Horwitz & Loeb 1988; Bagga *et al.* 1990). Our hypothesis was that the CHPS would extrude out to form a cruciform at a high supercoil density and inhibit transcription elongation. On a relaxed template, the sequence would collapse back into B-DNA conformation and allow the transcription machinery to go through. Thus, the CHPS would provide a relatively simple mechanism for the cell to sense supercoiling and couple it to transcription regulation. To test whether the promoter itself or regions in its immediate vicinity, including the CHPS, were responsible for RST, *M. smegmatis* cells harbouring the three short constructs (pSUN1, 3 and 5, Fig. 1C) were tested for their response to novobiocin. If

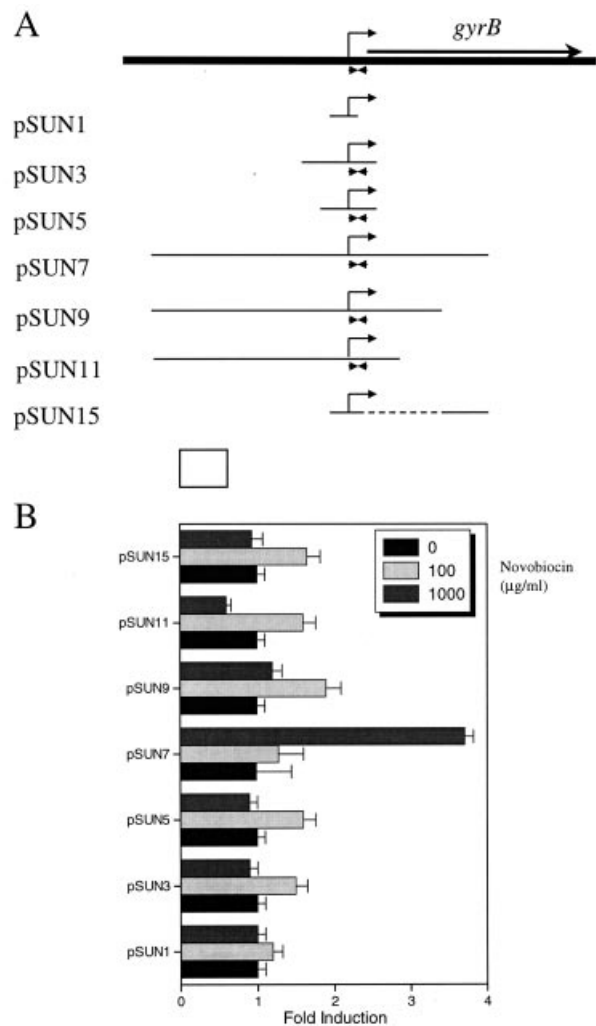


Figure 4 RST in the plasmid context. (A) All the constructs have been shown schematically. The transcription start site (\rightarrow) and CHPS (\leftrightarrow) are indicated. (B) Fold induction of CAT activity of cells harbouring various constructs after treatment with different amounts of novobiocin ($\mu\text{g}/\text{mL}$) for 4 h.

the RST were operational, it would be reflected in an increase in CAT specific activity. We found that there was no significant increase in CAT specific activity in any of these shorter constructs—comprising promoter and promoter proximal regions (Fig. 4B). We tested these cells with a large range of novobiocin concentrations (10 $\mu\text{g}/\text{mL}$ to 1000 $\mu\text{g}/\text{mL}$) to ensure that we were not missing out a particular window of sensitivity. The steady state levels of GyrA in these transformants showed a similar increase, as seen in Fig. 3. Quantitative ELISA revealed that induction of the genomic copy was unaffected by the presence of the short constructs.

Gene/promoter	- 35	- 10	Reference
<i>ask</i>	CCCACG	ACGCGT	Cirillo <i>et al.</i> (1994)
acetamidase	TTGACT	TATATT	Mahenthalingam <i>et al.</i> (1993)
<i>rmB</i>	TTGACT	TAACTT	EMBL acc. no. U09862
S4	GACCTT*	TATAAG	Bashyam <i>et al.</i> (1996)
S16	GCTTCG*	TATAAT	Bashyam <i>et al.</i> (1996)
S18	TCATTG*	TAAAGT	Bashyam <i>et al.</i> (1996)
S19	AAAGGC*	TAGTAT	Bashyam <i>et al.</i> (1996)
<i>gyr</i>	TCGGTG	TAGACT	This work

* Not proposed by Bashyam *et al.*; extrapolated from the sequence.

Table 2 Compilation of strong promoters from *M. smegmatis*

Role of distal regions in RST

There could be many reasons for the failure of short promoter fragments to respond to novobiocin in the plasmid context (see Discussion). One explanation is that the plasmid constructs lacked some DNA element that was located further away from the promoter that is essential for RST. To test this possibility, we created a construct containing a 2.5-kb *Bam*HI fragment (pSUN7) encompassing the promoter (Fig. 4A). As compared to the shorter constructs, pSUN7 is significantly stimulated by novobiocin (Fig. 4B). The 3.5-fold stimulation is comparable to the induction obtained in the genomic context. These cells respond only at a level of 1 mg/mL of novobiocin, probably because pSUN7 contains the N-terminal half of GyrB which is responsible for binding novobiocin. The over-expression of truncated fragments of GyrB have been shown to confer resistance to novobiocin in *E. coli* (del Castillo *et al.* 1991). To further delineate the elements that are responsible for RST, we made sequential deletions in the downstream region (pSUN9 and pSUN11; Fig. 4A). Both these deletions significantly lower the response to changes in supercoil levels (Fig. 4B). These experiments suggest that DNA elements present 600 bp downstream of the promoter play an essential role in RST in mycobacteria. However, the downstream element along with the minimal promoter region (pSUN15; Fig. 4A) is not sufficient to respond to novobiocin (Fig. 4B).

Discussion

We have presented the first clear demonstration of a dicistronic organization of the *gyr* genes in any species. The transcription is driven from a single promoter located upstream of *gyrB*. The promoter is specific to

mycobacteria and does not function in *E. coli*. We find that RST, although it is operational in *M. smegmatis*, appears to employ a different mechanism from *E. coli*.

The positioning of regulatory elements in the *gyr* locus points towards a co-ordinated expression of the two genes. RT-PCR with primers specific to *gyrA* and *gyrB* demonstrates that the genes are transcribed as a single operon. In addition, the only detectable promoter is located upstream of the *gyrB* (Fig. 2), while rho-independent terminator-like sequences are present exclusively downstream of *gyrA*. The short intergenic region lacks any such features. We find no transcription start site upstream of *gyrA*. Furthermore, the *gyrB* gene has a strong SD sequence (AGGAGA) upstream of a weak start codon (GTG), while *gyrA* has a poor SD sequence (AGGATT), but employs the more efficient ATG as the start codon. Such an arrangement suggests a translational coupling mechanism that could facilitate the production of equimolar amounts of the two subunits that would be required for making a functional enzyme. The genomic organization of *gyr* genes in many species also suggests an operon-like arrangement (Sugino & Bott 1980; Colman *et al.* 1990; Holmes & Dyall-Smith 1991; Thiara & Cundliffe 1993; Madhusudan *et al.* 1994; Salazar *et al.* 1996). However, it is noteworthy that in *Bacillus subtilis*, although the *gyrA* and *gyrB* genes are near each other, they are transcribed independently (Lampe & Bott 1985).

An analysis of the putative promoter elements of the *gyr* operon of *M. smegmatis* reveals that the -10 region resembles the consensus sequence for the σ^{70} promoters of *E. coli* (Hawley & McClure 1983) while the -35 region does not. A compilation of strong promoters identified in *M. smegmatis* is shown in Table 2. As can be seen, most of these promoters also show a greater conservation at the -10 region than the -35. In comparison with the promoters analysed by Dasgupta

et al. (1993), the *gyr* promoter is amongst the strongest to be isolated from *M. smegmatis*. The strongest promoter in their study has a CAT specific activity of less than 2500 nmol/min/mg, while cells harbouring pSUN3 show an activity of about 4300 nmol/min/mg. Thus, the *gyr* promoter has the potential to be used for homologous over-expression of genes in mycobacteria.

We have found that the *gyr* operon in *M. smegmatis* is induced by novobiocin at the transcriptional level (Figs 2A, 3). The presence of a strong CHPS (Fig. 1B) with an 8 base pair stem and a 4 base loop in the 5' UTR suggested a potential mechanism for RST in *M. smegmatis*. The *gyrA* gene from *Klebsiella pneumoniae* also has a cruciform potential sequence which overlaps with the -35 elements in the putative promoter which has been proposed to be involved in RST (Dimri & Das 1990). Similarly, cruciform potential sequences in the 5' UTR have been shown to affect transcription elongation (Bagga *et al.* 1990; Brahmachari *et al.* 1991). We find that the 5' untranslated region—including the CHPS—has a positive effect on promoter activity (Table 1) but is not sufficient for the operon to respond to novobiocin (Fig. 4). There could be a multitude of reasons for this observation. Firstly, a much higher level of novobiocin may be required to inhibit the mycobacterial gyrase and induce global relaxation. Secondly, a *trans* factor(s) may be involved in RST that is titrated out in a multicopy plasmid context. Thirdly, distal regions may be necessary for RST to occur. If the first possibility were true, then, in these cells, there would be no induction at the genomic level. In the second case, the *gyr* genes at the genomic level would be induced only in cells transformed with the vector pSD7 but not with any of the promoter constructs. The induction of the genomic copy and the results with the longer constructs (Fig. 4) essentially show the crucial role that is played by regions downstream of the promoter. We find DNA elements that are present 600-bp downstream of the promoter are necessary for RST to occur in the plasmid context. However, this region alone is not sufficient to confer sensitivity to supercoiling. The role played by distal elements in *M. smegmatis* may be direct (as a binding site for some *trans* factor) or indirect (as a modulator of supercoil levels). These results are in sharp contrast to the *gyr* genes in *E. coli* where the -10 region itself is responsible for RST, although it is devoid of any structure potential sequences (Straney *et al.* 1994). It is important to reiterate here that although extensive work has gone into localizing the phenomenon of RST to the -10 region of the *gyr* promoter in *E. coli*, a model at the molecular level is still lacking.

Thus, while RST makes biological sense, mechanistically it remains a mystery wherever it has been studied. It appears that although the phenomenon of autoregulation of *gyr* genes by sensing global supercoil levels is conserved in different species, the sensing mechanism itself differs. In *E. coli*, special features (if any) in the promoter itself, alone or along with a *trans* factor(s), seem to be involved in the process. *M. smegmatis*, on the other hand, appears to employ regions distant from the promoter. It is possible that the topological status of distant regions is measured and communicated to the promoter, influencing the transcriptional process itself.

Experimental procedures

Bacterial strains and plasmids

All the strains and plasmids used in this study are listed in Table 3. The *E. coli* strain DH10B was used for all cloning experiments and as the *E. coli* host for the CAT assays. *M. smegmatis* SN2 was used for the promoter mapping experiments. *M. smegmatis* mc²155 was used as the mycobacterial host for all the CAT assays. The *E. coli* cells were grown in Luria-Bertani (LB) medium, while the *M. smegmatis* cells were grown either in modified Youmans and Karlson's medium (Nagaraja & Gopinathan 1980), with 2% or 0.5% glycerol, or LB medium supplemented with 0.5% glycerol and 0.2% Tween-80. Kanamycin was added at 35 µg/mL where appropriate.

The promoter selection shuttle vector pSD7 (Dasgupta *et al.* 1993) was used for the promoter analysis. *E. coli* DH10B cells were transformed by the standard calcium chloride method (Sambrook *et al.* 1989). *M. smegmatis* cells were transformed as previously described (Dasgupta *et al.* 1993). After transformation, the cells were plated on LB agar containing 0.5% glycerol with kanamycin (35 µg/mL), either alone or in combination with chloramphenicol (25 µg/mL).

RNA isolation

For the S1 nuclease analysis, log-phase cultures of *M. smegmatis* SN2 were harvested, either directly or after two hours of treatment with novobiocin (25 µg/mL). The pellet was washed and resuspended in Buffer A (300 mM sodium acetate pH 4, 10 mM EDTA), and extracted with acid phenol at 65 °C. The cell suspension was sonicated to complete the lysis. After centrifugation, the aqueous layer was re-extracted with phenol and chloroform-isoamyl alcohol (24:1) and the RNA was finally precipitated with 2.5 volumes of ethanol and resuspended in DEPC-treated water. For RT-PCR and the primer extension analysis, RNA was isolated using TRIzol Reagent (Gibco BRL) following the manufacturer's instructions.

Table 3 Strains and plasmids

Strains/plasmids	Relevant features	Source/reference
<i>M. smegmatis</i> SN2	Wild-type	Laboratory stock
<i>M. smegmatis</i> mc ² 155	High efficiency transformation strain	Snapper <i>et al.</i> (1990)
<i>E. coli</i> DH10B	High efficiency transformation strain	Laboratory stock
<i>E. coli</i> NH647	gyrA ^{ts}	M. Gellert
<i>E. coli</i> N4177	gyrB ^{ts} cou ^r	N. R. Cozzarelli
pSD7	<i>E. coli</i> -mycobacteria shuttle promoter-selection vector with a promoterless CAT gene.	Dasgupta <i>et al.</i> 1993;
pMN9A	4.5-kb genomic fragment containing the upstream, entire gyrB and part of the gyrA gene in pUC19.	Madhusudan & Nagaraja 1995
pSUN1	Derivative of pSD7 with a 257-bp promoter fragment in the correct orientation (Figs 1C, 4)	This work
pSUN2	Same as pSUN1 but with the promoter in the opposite orientation (Fig. 1C).	This work
pSUN3	Derivative of pSD7 with a 377-bp promoter fragment in the correct orientation (Figs 1C, 4).	This work
pSUN4	Same as pSUN3 but with the promoter in the opposite orientation (Fig. 1C).	This work
pSUN5	Derivative of pSD7 with a 317-bp promoter fragment in the correct orientation (Figs 1C, 4).	This work
pSUN6	Same as pSUN5 but with the promoter in the opposite orientation (Fig. 1C).	This work
pSUN7	Derivative of pSD7 with a 2.5-kb promoter fragment in the correct orientation. The fragment contains 1.5 kb upstream and 1 kb downstream of the promoter (Fig. 4).	This work
pSUN9	Derivative of pSUN7 with the last 390 bp deleted from the downstream segment (Fig. 4).	This work
pSUN11	Derivative of pSUN7 with the last 790 bp deleted from the downstream segment (Fig. 4).	This work
pSUN15	Derivative of pSUN1 with the last 390 bp from the downstream segment of pSUN7 added (Fig. 4).	This work

Primer extension and RT-PCR

Primer extension was performed as per the manufacturer's instructions using Superscript II reverse transcriptase (Gibco BRL) using primers P_C or P_A. The primers were previously end-labelled using γ -P³²-ATP (> 5000 Ci/mol, Amersham) and T4 polynucleotide kinase (Gibco BRL). For RT-PCR, first strand synthesis was performed with Superscript II reverse transcriptase (RT) using P_A. The PCR was carried out with P_A and P_B using *Taq* polymerase in two parts—for the first five cycles, the annealing was at 40 °C, followed by 25 cycles with annealing at 55 °C.

P_A: 5' TCG ACC GGT TCG ATC CGG TC 3'

P_B: 5' GTT GTT GGG CCG GTT CAT GAA GC 3'

P_C: 5' TCG AGA ATG GTG ATG GAA TCG GC 3'

S1 nuclease analysis

The S1 nuclease mapping method was adapted from Sharp *et al.* (1980). A 600-bp *NruI*-*XhoI* fragment spanning the putative start codon for *gyrB* was used as the probe (Fig. 1). This fragment was

radioactively labelled at the *XhoI* site using γ -³²P-ATP and T4 polynucleotide kinase. Three hundred μ g of RNA were co-precipitated with 2×10^5 c.p.m. and heated at 97 °C for 10 min in 25 μ L of hybridization buffer (40 mM piperazine-N,N'-bis(2-ethanesulphonic acid) pH 6.5, 400 mM sodium chloride, 1 mM EDTA, 80% formamide) and incubated at 65 °C for 16–20 h. 400 μ L of S1 nuclease buffer (30 mM sodium acetate pH 4.5, 300 mM sodium chloride, 1 mM zinc sulphate) was added and 200 μ L aliquots were digested with different concentrations of S1 nuclease (200 U and 400 U) for 45 min at 37 °C. The protected fragments were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated. The pellet was dissolved in formamide loading dye and analysed on an 8 M urea–6% polyacrylamide gel. The DNA sequence ladder was generated using the primer P_C—designed to the *XhoI* end.

CAT assays and novobiocin treatment

For determination of the CAT-specific activity, a single colony was inoculated into 3 mL of medium and grown to mid-log

phase. The cells were harvested, washed with an equal volume of Buffer B (250 mM Tris-HCl, pH 8) and resuspended in 1/4 volume of the same buffer. The cells were sonicated and centrifuged at 15000g for 15 min at 4°C. An appropriate dilution of the supernatant was assayed for CAT activity. The reaction contained 100 µM ¹⁴C-chloramphenicol (5 mCi/mmol) and 300 µg/mL acetyl coenzyme A in 250 mM Tris-HCl (pH 8). The reaction was carried out at 37°C for 30 min and stopped with 10 volumes of cold ethyl acetate. The organic layer was dried. Samples were resuspended in a minimal volume of ethyl acetate and resolved on a fluorescent silica thin layer chromatography (TLC) plate with chloroform:methanol (95:1). After autoradiography, the region corresponding to the products and substrate was cut out and its radioactivity was measured in a liquid scintillation counter. The CAT activity was calculated as the percentage of the substrate converted to monoacetylated form in 30 min at 37°C per 100 ng of the protein.

For induction by novobiocin, cells were grown to mid-log phase in 30 mL of starvation medium. The cultures were distributed into six tubes with 5 mL in each, treated with different concentrations of novobiocin, and allowed to grow further. 900 µL aliquots were taken every hour and frozen in liquid nitrogen at -70°C. When required, these aliquots were thawed on ice and processed as described above.

Immunoblot analysis and ELISA

For immunoblotting, 6 µg of the crude cell extract was resolved on a 1% SDS-8% PAGE and electroblotted on to a polyvinylidene difluoride membrane. The blot was probed with polyclonal antibodies (1:5000) raised in rabbit against *M. tuberculosis* GyrA. The blot was developed with secondary antibody conjugated with horseradish peroxidase (1:2000, Sigma) and 3-amino-9-ethylcarbazole (Sigma). For the ELISA, 10 µg of the crude cell extract was coated on to each well. The plates were probed as described above and developed with tetramethylbenzidine.

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