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DNA Gyrase Genes in *Mycobacterium tuberculosis*: a Single Operon Driven by Multiple Promoters

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The two genes encoding DNA gyrase in *Mycobacterium tuberculosis* are present next to each other in the genome, with *gyrB* upstream of *gyrA*. We show that the primary transcript is dicistronic. However, in addition to the principal promoter, there are multiple weaker promoters that appear to fine-tune transcription. With these and other mycobacterial promoters, we propose consensus promoter sequences for two distinct sigma factors. In addition to this, the *gyr* genes in *M. tuberculosis*, as in other species, are subject to autoregulation, albeit with slower kinetics, probably reflecting the slower metabolism of the organism.

Most of our understanding of prokaryotic transcription initiation is based on extensive analysis of promoter architecture in *Escherichia coli*. Since the regions in σ^{70} involved in contacting the promoter show extensive conservation across the prokaryotic world (13), a similar picture for transcription initiation is expected in all bacteria. However, this does not appear always to be the case. For instance, results of earlier random promoter screens indicate that only a small fraction of mycobacterial promoters are recognized by the *E. coli* machinery (6, 26). Furthermore, a random promoter screen in *Mycobacterium paratuberculosis* detected only promoters that were highly GC-rich in both their −10 and −35 regions (2). Thus, the features that define species-specific promoters are not clear.

Here we present the analysis of the transcription of the DNA gyrase genes in *Mycobacterium tuberculosis*. As the sole supercoiling activity in the cell, DNA gyrase faces the daunting task of opposing the relaxing activities of both topoisomerases I and IV (29). As a result, DNA gyrase is essential in all eubacterial cells that have been tested so far, and the final topology of DNA is maintained by the equilibrium achieved by these divergent forces. Since DNA gyrase needs to oppose the relaxation induced by other topoisomerases, it regulates its own synthesis by a unique mechanism. In general, transcription of most genes is induced by increased negative supercoiling. In contrast, negative supercoiling represses transcription of the gyrase genes in *E. coli* (15). This phenomenon, referred to as relaxation-stimulated transcription, is believed to be the cell's strategy to homeostatically maintain the topology of DNA (15). Thus, increased gyrase levels lead to an increase in supercoiling, which, in turn, repress the expression of gyrase and allow other topoisomerases to bring the topology of the DNA back to its optimum state. Relaxation-stimulated transcription appears to be conserved in all organisms tested so far (14, 23, 25, 28); however, the underlying mechanism appears to vary (27).

Therefore, there are multiple reasons to analyze the transcription of the *gyr* genes in *M. tuberculosis*, especially since the genome lacks both topoisomerases III and IV (5). In addition, since the expression of many virulence genes is dependent on the topology of DNA in many pathogenic bacteria (8), understanding the regulation of DNA gyrase in *M. tuberculosis* might help decipher the various players involved in the infection process. Our analysis revealed that while the majority of the *gyr* message is dicistronic, additional promoters are present that appear to be regulatory in function. From these as well as other promoters identified previously in mycobacteria, we have developed two potential consensus sequences specific to mycobacterial promoters. In addition to this, we found that although the *gyr* genes were subject to relaxation-stimulated transcription, the kinetics of the process was significantly slower than in other species such as *E. coli* and *Mycobacterium smegmatis*, probably reflecting the overall slow metabolism of the organism.

MATERIALS AND METHODS

Bacterial strains and transformation. *E. coli* strain DH10B was used for all cloning experiments and as the *E. coli* host for the chloramphenicol acetyltransferase (CAT) assays. *M. tuberculosis* H37Ra was used for the promoter mapping experiments. *M. smegmatis* mc^{2}155 was used as the mycobacterial host for all the CAT assays. The *E. coli* cells were grown in Luria-Bertani (LB) medium. The mycobacterial cells were grown in modified Youmans and Karlson's medium (17) with 2% glycerol and 0.2% Tween 80. Kanamycin was added at 35 μg/ml where appropriate. The *E. coli* DH10B cells were transformed by the standard calcium chloride method (22). The *M. smegmatis* cells were transformed as described before (7). 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, RT-PCR, and primer extension. For RNA isolation, *M. tuberculosis* cells were grown for 15 days with intermittent shaking (≈1.0 optical density unit at 600 nm), harvested, and resuspended in Trizol reagent (Gibco-BRL). RNA was isolated as described previously (28). Primer extension was performed with Superscript II reverse transcriptase (Gibco-BRL) with appropriate primers (primer A for P_A, B for P_{B1}, and R for P_R). Briefly, 2 μg of total RNA was mixed with 10 pmol of end-labeled specific primer, denatured at 95°C for 10 min, and quick chilled on ice immediately. After adding the reaction buffer, deoxynucleoside triphosphates (500 μM each), 10 mM dithiothreitol, and 10 U of pancreatic RNase inhibitor (Gibco-BRL), samples were incubated at

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50°C for 2 min. The reaction was started with the addition of 200 U of Superscript II.

For reverse transcription (RT)-PCR, first-strand synthesis was performed with Superscript II reverse transcriptase and primer A as described above. Then 1/10th of the reaction was subjected to PCR with primers A and C with *Taq* polymerase, in two parts. For the first five cycles, the annealing was at 45°C, followed by 25 cycles with annealing at 55°C. The primer A sequence was 5′-TCGACCGGTTCGATCCGGTC-3′, that of primer B was 5′-CACCATGA ATTCCTCGGTTCGTGTG-3′, that of primer C was 5′-CAGCCACGATCCG AATACTC-3′, and that of primer R was 5′-CGAAGCGAATTCGTATGCCG GACGTC-3′.

For induction by novobiocin, *M. tuberculosis* cells were grown for 15 days with intermittent shaking. Cultures were shifted to a water bath for continuous shaking. After allowing 24 h for adaptation, the cells were treated with 100 μg (final concentration) of novobiocin per ml. Aliquots were taken every 12 h, harvested, resuspended in Trizol reagent (Gibco-BRL), frozen in liquid nitrogen, and stored at −70°C. RNA was isolated as described previously (28).

DNA manipulation. Putative promoter fragments were cloned at the *Bam*HI site in the promoter selection shuttle vector pSD7 (7) for testing promoter strengths. pTUN1 and pTUN2 contain a 1.5-kb *Bam*HI fragment from the region upstream of *gyrB*. pTUN3 and pTUN4 contain a 1.5-kb *Bam*HI fragment including 100 bp upstream of *gyrB* and 1.4 kb of the *gyrB* gene itself. pTUN5 and pTUN6 contain a 900-bp *Bam*HI fragment that includes 200 bp upstream of *gyrA* and 700 bp of the *gyrA* gene itself. All odd-numbered clones have the promoter elements in the correct orientation, while the even-numbered clones have them in the reverse orientation.

CAT assays and immunoblot analysis. CAT assays were performed with exponentially growing *M. smegmatis* cells as described previously (28). For immunoblotting, 10 μg of the crude cell extract was resolved by 1% sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane. The blots were probed with polyclonal antibodies (1:5,000) raised in rabbit against *M. tuberculosis* GyrA or GyrB. The blot was developed with secondary antibody conjugated with horseradish peroxidase (1:2,000; Sigma Chemicals). For GyrA, 3-amino-9-ethylcarbazole was used as the substrate, and for GyrB, the ECL-Plus system (Amersham Pharmacia Biotech) was used.

Sequence analysis. To develop a consensus for promoter elements for mycobacteria, 82 promoters for which the transcription start site had been experimentally defined were selected from the literature. Individual promoters were iteratively clustered into multiple groups. The final two groups of promoters included 80 of these promoters. The frequency of occurrence of different bases at individual positions was used to generate a consensus matrix. From this matrix, a simplified consensus was developed by selecting bases that were statistically overrepresented. Overrepresentation was determined by performing a χ^2 test while taking into account the high GC content of mycobacterial genomes. Therefore, for instance, a 30% occurrence would not be considered significant for a G or C at a given position but would be considered significant for an A or T. The entire list of mycobacterial promoters and their analysis is available in the form of supplementary material upon request.

RESULTS

Mapping the transcription start site in the *gyr* locus. The active DNA gyrase is composed of two subunits, GyrA and GyrB, products of separate genes, that form an A_2B_2 heterotetramer (20). The genomic arrangement of the genes that encode these two subunits varies greatly among different bacteria. For instance, in *E. coli*, while the *gyrB* gene is close to *oriC*, *gyrA* is almost at the diametrically opposite end (1). On the other hand, the genomes of many gram-positive organisms, including several mycobacterial species, have the two genes close to each other near *oriC*, with *gyrA* present downstream of *gyrB* in the vicinity of the chromosomal origin of replication (21). However, despite their proximity, in some organisms such as *Bacillus subtilis*, the two genes are transcribed independently (12), while in others, such as *Borrelia burgdorferi* (11) and *M. smegmatis*, they are part of a single dicistron (28).

The *gyr* genes in *M. tuberculosis* are located close to the origin of replication, with *gyrB* present 34 nucleotides upstream of *gyrA* (24). The short intergenic region is devoid of promoter or terminator-like features, implying that the genes are part of a single transcript, as in *M. smegmatis* (28). To identify the potential promoters upstream of *gyrA* and *gyrB*, primer extension analysis was performed with primers specific to *gyrA* and *gyrB* (Fig. 1). In contrast to the result with *M. smegmatis*, both reactions generated specific products, indicating that each gene is transcribed independently by its own promoter, located approximately 60 nucleotides upstream of the respective start codons. Interestingly, the putative promoter elements of the two genes were strikingly different (discussed later).

Promoter activity in *E. coli* and *M. smegmatis*. To functionally test these promoters and to determine their relative strengths, fragments encompassing each promoter (Fig. 2) were cloned in the promoter selection vector pSD7 (7). All *E. coli* transformants were sensitive to chloramphenicol, and cell extracts from these transformants did not show any detectable CAT activity. On the other hand, the *M. smegmatis* transformants were resistant to chloramphenicol to different extents (compare pTUN3 and pTUN5 in Fig. 2). These results were paralleled by the specific CAT activity of these constructs, with the P_{B1} promoter fragment showing approximately 70-fold higher activity than P_A (Fig. 2). However, in addition, there were some surprising results. First, while the fragment containing P_A showed strict orientation-dependent expression (compare pTUN5 and pTUN6, Table 1), the fragment containing P_{B1} showed expression even in the reverse orientation, albeit at a lower level than in the correct orientation (compare pTUN3 and pTUN4, Fig. 2). The activity in the reverse orientation was about 13-fold weaker than that of P_{B1} and 5.5-fold stronger than that of P_A. In addition, a fragment corresponding to a region upstream of P_{B1} (pTUN1 and pTUN2, Fig. 2) showed orientation-dependent expression comparable to that of P_A (Fig. 2).

The transcription start corresponding to the promoter in the reverse orientation (P_R) was mapped with RNA isolated from *M. tuberculosis* (Fig. 3). The position of the transcription start site implies that P_R substantially overlaps P_{B1}. Repeated attempts to precisely locate the upstream promoter activity (P_{B2}) by primer extension failed, probably due to its weak nature. To further substantiate the results from the functional assay, primer extension analysis was carried out with RNA isolated from *M. smegmatis* cells transformed with appropriate constructs. The transcription start sites for P_{B1} and P_R mapped to the same position as obtained with RNA from *M. tuberculosis* (not shown). However, not surprisingly, due to its weak activity, the transcription start site corresponding to P_A could not be detected in *M. smegmatis*.

Organization of *gyr* genes in *M. tuberculosis*. The presence of a promoter specific to *gyrA* itself raised the possibility that the two *gyr* genes of *M. tuberculosis* were transcribed independently, unlike the dicistronic arrangement in *M. smegmatis*. On the other hand, P_{B1} was 70-fold stronger than P_A, at least when tested in *M. smegmatis*, indicating that it could be the primary initiation site for both genes. To ascertain whether the *gyr* genes were part of a single dicistron, RT-PCR was performed with *M. tuberculosis* RNA and primers specific to *gyrA* and *gyrB*. These showed specific amplification of a 240-bp product encompassing the intergenic region, proving that at least the primary transcript was dicistronic.

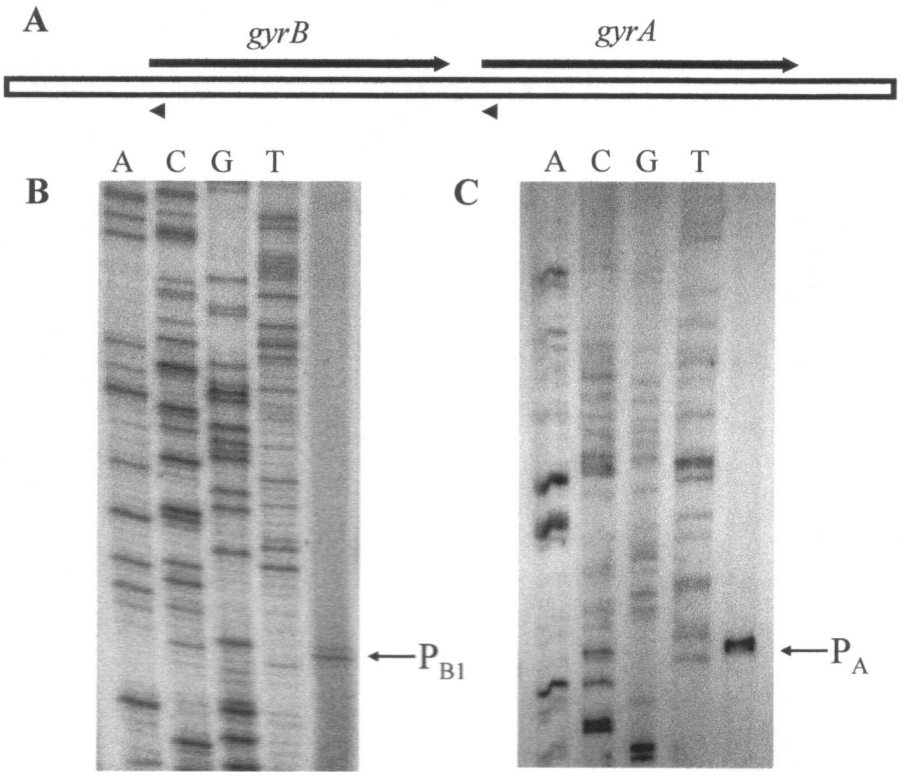
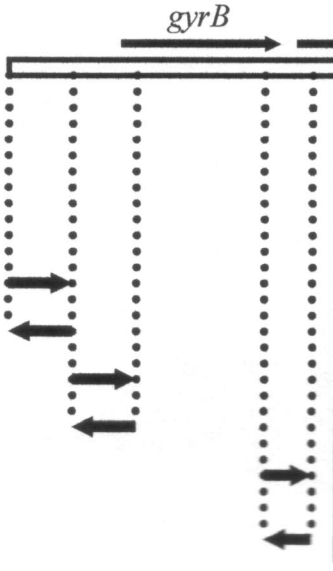


FIG. 1. Primer extension analysis to map transcription start sites upstream of *gyrB* (B) and *gyrA* (C). (A) Schematic of the *gyr* operon in *M. tuberculosis*. Arrowheads represent the primers used for the analysis. The extension product corresponding to the transcription start site for each promoter is indicated. The sequencing lanes were used as markers. RNA was prepared from exponentially growing *M. tuberculosis* cells.

Autoregulation of DNA gyrase. The presence of multiple promoters appeared to suggest complex regulation of the *gyr* genes in *M. tuberculosis*. However, because it is a slow-growing organism, we expected it to be more tolerant of changes in topology than faster-growing species, probably producing a more subtle response spread over a longer duration. In accordance with this expectation, when global relaxation was induced in *M. tuberculosis* cells by novobiocin treatment (9),



Plasmid	cfu (x 10^4)			CAT specific activity (U)
	Kn^r	Chl^r (12.5)	Chl^r (25)	
pSD7	4.1	0	0	11.1
pTUN1	4.0	3.5	0	106.1
pTUN2	3.4	0	0	ND
pTUN3	4.3	4.5	4.4	11962.0
pTUN4	3.1	3.7	3.0*	916.3
pTUN5	4.3	4.1	0	176.4
pTUN6	3.5	0	0	ND

FIG. 2. Functional analysis of putative promoters in *M. smegmatis*. The arrows denote the orientation of the clone. cfu denotes CFU obtained on plates containing either kanamycin alone (Kn^r) or with 12.5 or 25 μg of chloramphenicol (Chl^r) per ml, as indicated. *, slow-growing colonies. ND, not determined.

TABLE 1. Representative promoters from each class

Promoter	Species	Gene	Sequence					% Match
			−35	Spacer (nt)	−10	Spacer	+1	
SigA	*M. smegmatis*	*ace*	TTGACT	16	TATATT	6	G	92
	M. tuberculosis	*rrnA* P3	TTGACT	18	TAGACT	6	G	92
	L5	P_{left}	TTGACA	18	CATTCT	6	A	83
	M. fortuitum	*rrnA* P3	TTGACA	18	TAAGCT	6	G	83
	M. leprae	16S rRNA	TTGACT	16	ATTAAT	7	G	83
	M. paratuberculosis	PAN	TCGACA	17	TACACT	7	A	83
	M. phlei	*rrnA* PCL1	TTGACG	18	TAGACT	6	G	83
	M. smegmatis	*rrnA* P3	TTGACA	18	TAAGCT	6	G	83
	M. smegmatis	*furA*	TTGACT		TAGCCT			83
	M. tuberculosis	*furA*	TTGACT		TATTGT			83
SigGC	*M. paratuberculosis*	pAJB303	TGGCGT	16	CGGCAC	7	T	92
	M. paratuberculosis	pAJB73	TGCCGC	20	CTCCAG	7	T	83
	M. paratuberculosis	pAJB86	TGACGT	17	CGGTCC	6	T	83
	M. paratuberculosis	pAJB300	TGACGC	17	CAGCCG	7	A	83
	M. bovis	*mpb70*	TACCGA	19	CATCAG	6	G	75
	M. paratuberculosis	pAJB301	TCCAGT	20	CTGGCC	7	T	67
	M. tuberculosis	85A	TACACG	17	CGCCTG	7	A	58
	M. paratuberculosis	pAJB305	TGTTGG	17	TGGTTG	7	T	50
	M. tuberculosis	*katG* PC	TTCGCG	14	CACAGC	7	C	50
	M. tuberculosis	*cpn60*	TGCTCA	17	GGCGGC	7	A	50
	M. tuberculosis	*gyrA*	CGACGC	17	CCCGCA	7	G	50

there was little change in the steady-state level of either GyrA or GyrB up to 12 h. It should be noted that much shorter durations are sufficient to induce relaxation-stimulated transcription in *E. coli* and *M. smegmatis* (15, 28). However, treatment for longer durations results in a time-dependent increase in both GyrA and GyrB, as in *M. smegmatis*, except over a long time period (Fig. 4). This induction is reflected at the level of transcription from the P_{B1} promoter (Fig. 5). Thus, the phenomenon of relaxation-stimulated transcription is conserved in *M. tuberculosis* as well, although the kinetics of induction are significantly slower. Concomitant to this induction, there was a decrease in transcription from the divergently organized P_R. In addition, a small yet reproducible decrease in transcription from P_A, the *gyrA*-specific promoter, was observed.

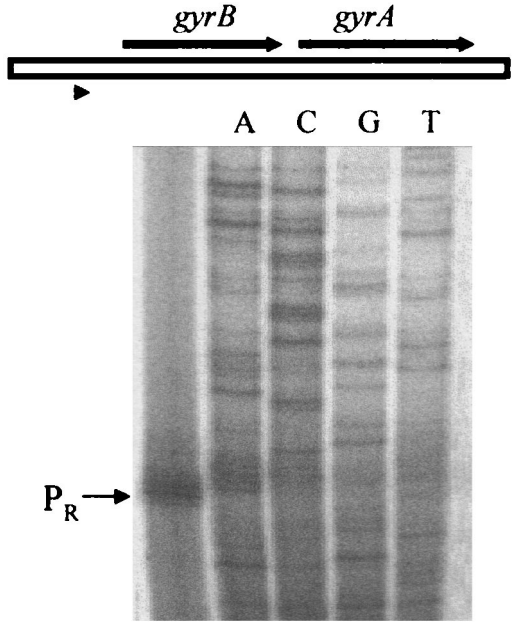


FIG. 3. Primer extension to map transcription start site corresponding to the reverse promoter. The extension product corresponding to the transcription start site for each promoter is indicated. The sequencing lanes were used as markers. RNA was prepared from exponentially growing *M. tuberculosis* cells.

DISCUSSION

A comparison of the expression of DNA gyrase in *M. smegmatis* and *M. tuberculosis* reveals an amalgamation of conserved and divergent features. The genomic arrangement of the *gyr* locus is substantially conserved between the two mycobacterial species, and the primary transcript is dicistronic in both species. In addition, the primary promoter in *M. tuberculosis*, P_{B1}, is located upstream of the *gyrB* gene at a position similar to that of the *M. smegmatis gyr* promoter. Furthermore,

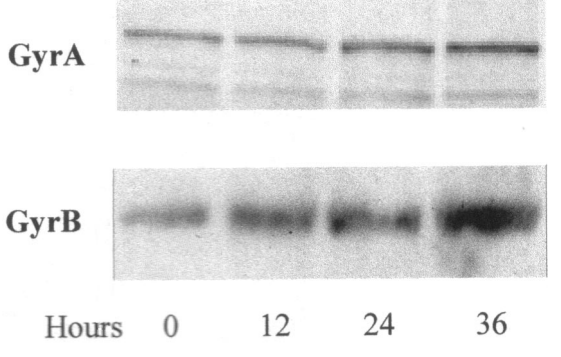


FIG. 4. Increase in DNA gyrase protein level in response to novobiocin. Western blot analysis of GyrA and GyrB with polyclonal antibodies raised against the individual proteins. Protein extracts were prepared after treatment of cells for the indicated durations with 100 μg of novobiocin per ml.

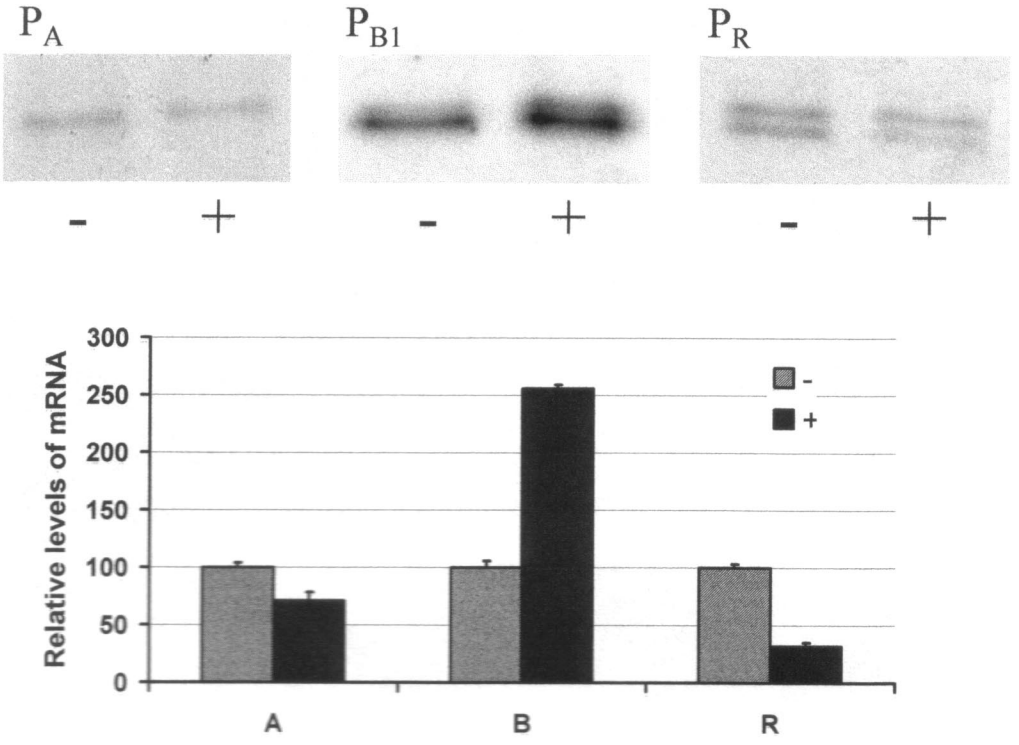


FIG. 5. Induction of the *gyr* transcript in response to novobiocin. Primer extension analysis was used to assess changes in the levels of transcript from P_A, P_{B1}, and P_R. RNA was prepared from cells before (−) and after (+) 12 h of treatment with 100 μg of novobiocin/ml. The primers illustrated in Fig. 1 and 3 were used for primer extension analysis.

the promoter region per se for P_{B1} shows extensive conservation with P_{gyr}, the promoter driving the *gyr* genes of *M. smegmatis* (Fig. 6), indicating that they are evolutionarily related.

Apart from the primary promoter, the *gyr* locus in *M. tuberculosis* employs at least two other promoters (Fig. 6). These additional promoters are weak and probably play a regulatory role. P_A is 70-fold weaker than P_{B1} in exponentially growing *M. smegmatis*. Therefore, it is unlikely to contribute greatly to the steady-state levels of the GyrA protein. On the other hand, it is possible that it employs an *M. tuberculosis*-specific σ factor or regulatory protein that is absent in *M. smegmatis*. Moreover, P_A may be induced under specific conditions which require only the production of excess GyrA. For instance, there is at least one report of induction of GyrA alone in *E. coli* in response to treatment with GyrA inhibitors (18).

The other weak promoter, P_R, is divergently oriented and almost completely overlaps P_{B1}. Therefore, the binding of RNA polymerase to one of them would prevent binding in the opposite orientation. It should be noted that there are no identifiable coding sequences upstream of *gyrB* that P_R could be involved in transcribing. Thus, the function of P_R is also likely to be purely regulatory. Overlapping, mutually exclusive promoters are one mechanism for regulating gene expression (16). For instance, recruitment of the polymerase to P_R would decrease expression of DNA gyrase by reducing transcription initiation. In the converse scenario, as in relaxation of the template, P_R is repressed and P_{B1} gets induced to almost the same extent.

Another point of interest was that all these promoters showed no detectable activity in *E. coli* while showing a wide range of activity in mycobacteria. The identification of promoters that function only in mycobacteria raised the possibility of defining features that are specific to mycobacterial transcription initiation. Sequence analysis revealed that the putative promoter elements of P_A do not follow the *E. coli* σ^{70} promoter consensus (Fig. 6). On the other hand, P_{gyr} from *M. smegmatis* along with P_{B1} and P_R from *M. tuberculosis* show moderate resemblance to the *E. coli* consensus (Fig. 6).

To develop a general consensus matrix for promoter elements for mycobacterial promoters, we analyzed 82 mycobacterial promoters for which the transcription start site had been experimentally defined. A majority of these promoters approximate the *E. coli* consensus to various extents. However, a subset of promoters, including P_A, have extremely GC-rich −10 and −35 regions. Therefore, we clustered the promoters iteratively into two classes. The consensus elements (Fig. 7) for the two classes of promoters are shown, along with representative members (Table 1). The complete consensus matrices as well as the classification of all promoters are included as supplementary material. These two classes encompass 80 of the 82 promoters used in the analysis. The only promoters that were excluded were two extremely weak promoters identified in *M. paratuberculosis* (2).

The major class includes 69 promoters that show considerable resemblance to the *E. coli* σ^{70} consensus (10). Since all residues known to be involved in base-specific contact of the promoter are conserved between *E. coli* and mycobacterial σ^{70} (3, 4), these promoters are probably recognized by SigA, the

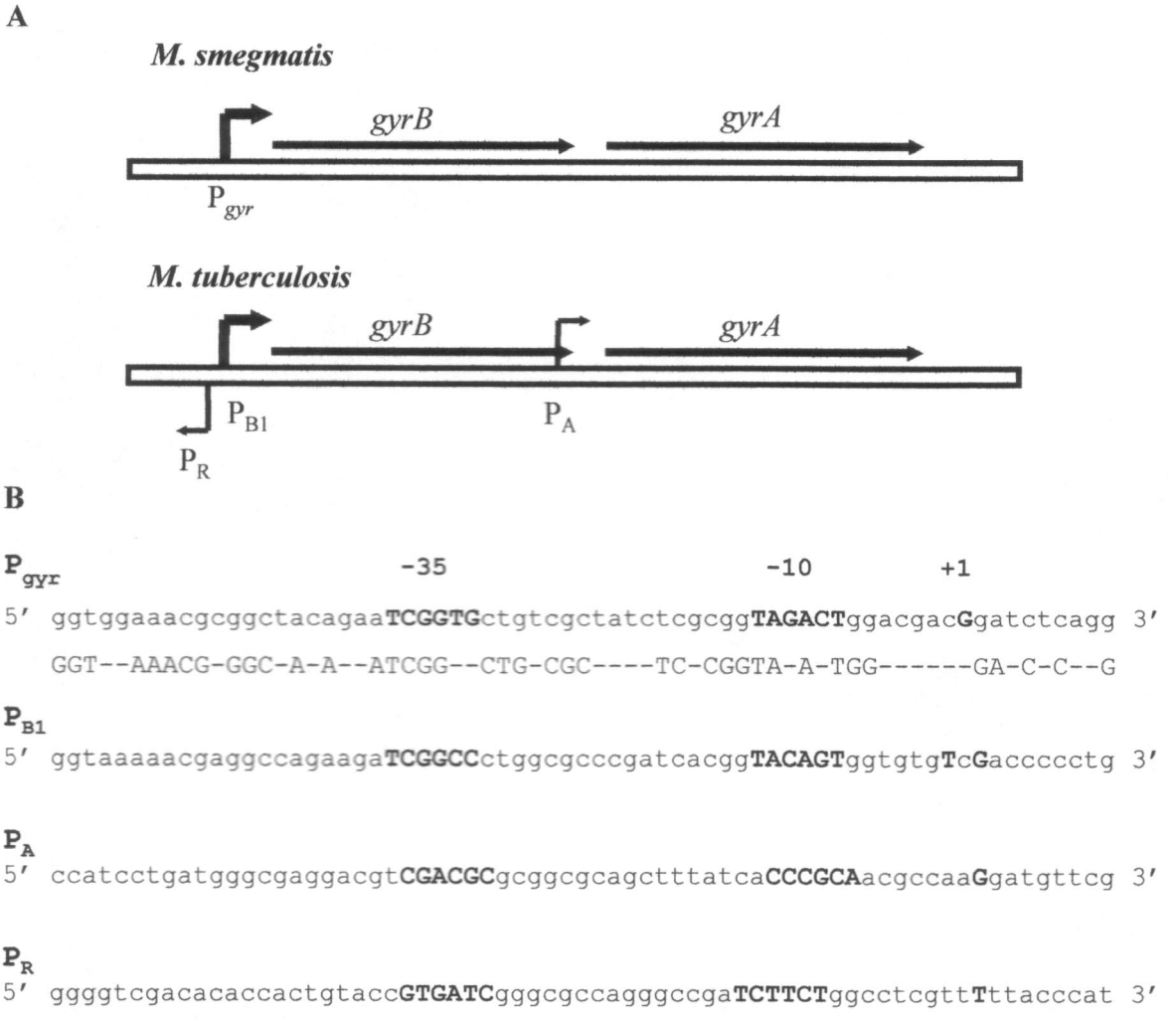


FIG. 6. Promoters in *M. smegmatis* and *M. tuberculosis*. (A) Comparison of the *gyr* locus in *M. smegmatis* and *M. tuberculosis.* (B) Putative promoter elements of the *gyr* promoters in the two species. The putative elements and transcription start sites are in bold uppercase letters. The sequence conservation between the primary promoters in the two species is also shown.

principal sigma factor in mycobacteria. However, it is not clear what additional features of these promoters make them functional in mycobacteria while still, by and large, being nonfunctional in *E. coli*.

On the other hand, the second class of 11 promoters represent a completely mycobacterium-specific consensus distinct from any promoter consensus reported in any organism so far. However, further work is required to identify which of the 13 sigma factors in *M. tuberculosis* recognizes this class of promoters (the putative SigGC). Since the representative promoter of this class (*gyrA* promoter from *M. tuberculosis*, P_A) is also recognized in *M. smegmatis*, the completion of the *M. smegmatis* genome sequence would provide some clues to SigGC. It is noteworthy that in both classes of promoters, there is a correlation between the strength of a promoter and how closely it approximates the consensus. For instance, among the putative SigA-driven promoters, those that show the closest match to the consensus include some of the strongest mycobacterial promoters (S16, *ace*, and *rrnA* promoters in Table 1). In addition, among the putative SigGC-driven promoters, the promoter that most closely resembles the consensus is the strongest promoter (pAJB303 in Table 1) identified in the random promoter screen by Bannantine et al. (2). Furthermore, the six promoters from their study that fall into this class show a correlation between their strength and the extent to which they approximate the consensus (Table 1). This is reminiscent of the *E. coli* σ^{70} paradigm, for which there is a similar correlation and the consensus promoter actually shows maximal activity (10).

Finally, as discussed above, *M. tuberculosis* appears to respond to relaxation of the genome by altering the utilization of promoters leading to increased synthesis of DNA gyrase. Therefore, the phenomenon of relaxation-stimulated transcription appears to be conserved in *M. tuberculosis*. However, the kinetics of response is extremely slow compared to that in *E. coli* or *M. smegmatis. E. coli* shows maximal response in as little as 5 min after treatment with novobiocin (15), while *M. smegmatis* takes 3 h (28). In contrast, *M. tuberculosis* takes over 24 h to reach a peak. However, independent of the time taken, all species appear to increase the level of protein by about

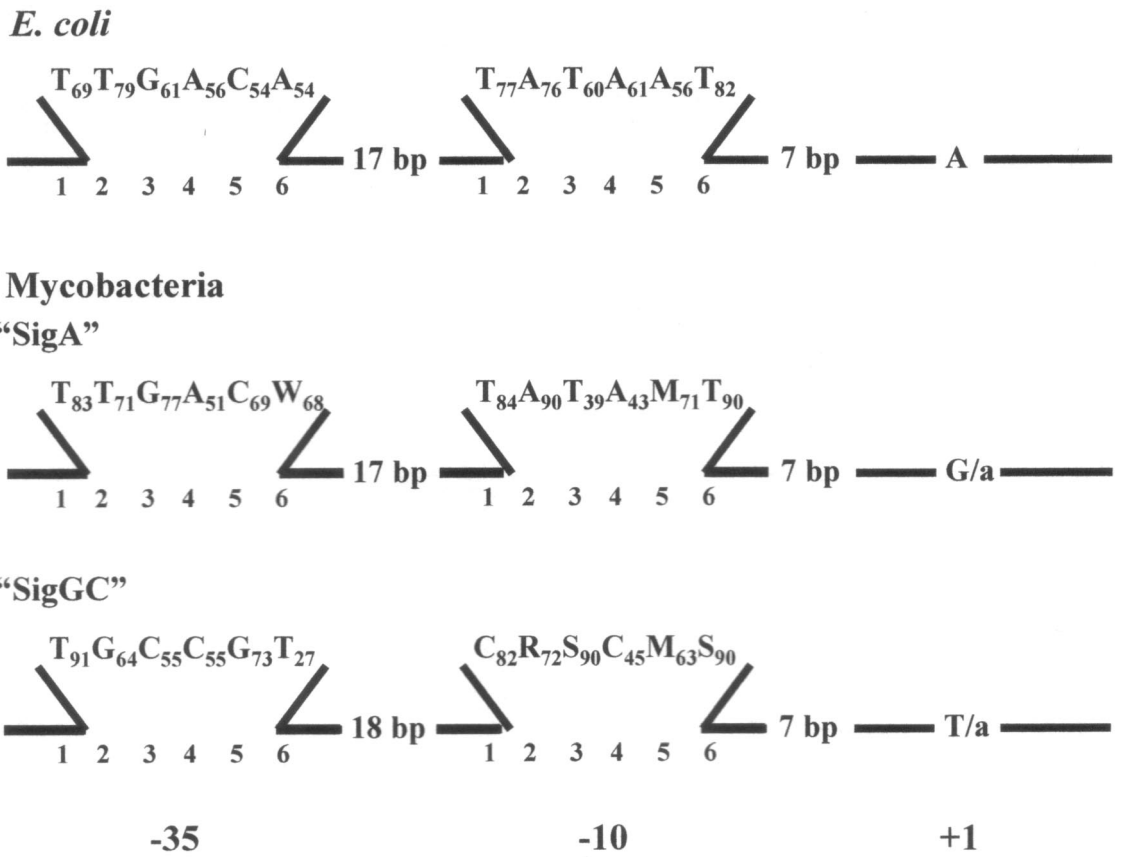


FIG. 7. Comparison of promoter consensus between *E. coli* and mycobacteria. The most frequently occurring bases at each position are listed. The number in the subscript denotes the frequency of occurrence. R denotes A or G, S denotes C or G, M denotes A or C, and W denotes A or T. The base numbering reflects the numbering used in the consensus matrix in the supplementary material (available on request).

threefold. Papavinasasundaram and coworkers reported a slow response in the induction of RecA in mycobacteria (19). Thus, the kinetics of the response seems to parallel the rate of growth and metabolism of each organism.

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