JOURNAL OF BACTERIOLOGY, June 1996, p. 3564–3571 Vol. 178, No. 12
0021-9193/96/$04.00+0

Genetic Analysis of the *Mycobacterium smegmatis rpsL* Promoter

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Received 29 January 1996/Accepted 9 April 1996

The DNA sequence of the promoter region of the *Mycobacterium smegmatis rpsL* gene, which encodes the S12 ribosomal protein, was determined. Primer extension analysis and S1 nuclease protection experiments identified the 5′ end of the *rpsL* mRNA to be 199 bp upstream of the translation initiation codon. The *rpsL* promoter contained sequences upstream of this start point for transcription that were similar to the canonical hexamers found at the −10 and −35 regions of promoters recognized by Eσ^{70}, the major form of RNA polymerase in *Escherichia coli*. To define the promoter of the *rpsL* gene, DNA fragments containing progressive deletions of the upstream region of the *rpsL* gene were inserted into a plasmid vector containing a promoterless *xylE* gene. These insertions revealed that the 200 bp of DNA sequence immediately upstream from the translation initiation codon was not essential for promoter function. In addition, 5′ deletions removing all but 34 bp upstream of the transcription start point retained greater than 90% promoter activity, suggesting that the −35 hexamer was not essential for promoter activity. To determine which nucleotides were critical for promoter function, oligonucleotide-directed mutagenesis and mutagenic PCR amplification were used to produce point mutations in the region upstream of the start point of transcription. Single base substitutions in the −10 hexamer, but not in the −35 hexamer, severely reduced *rpsL* promoter activity in vivo. Within the −10 hexamer, nucleotide substitutions causing divergence from the *E. coli* σ^{70} consensus reduced promoter activity. The DNA sequence immediately upstream from the −10 hexamer contained the TGn motif described as an extended −10 region in prokaryotic promoters. Mutations in this motif, in combination with a transition at either the −38 or −37 position within the −35 hexamer, severely reduced promoter activity, indicating that in the absence of a functional −35 region, the *rpsL* promoter is dependent on the TGn sequence upstream from the −10 hexamer. Comparison of the nucleotide sequence of the *rpsL* promoter region of *M. smegmatis* with the homologous sequences from *Mycobacterium leprae*, *Mycobacterium bovis*, and *Mycobacterium tuberculosis* showed the presence in these slowly growing mycobacterial species of conserved promoter elements a similar distance upstream of the translation initiation codon of the *rpsL* gene, but these other mycobacterial promoters did not contain the extended −10 motif.

The genus *Mycobacterium* comprises acid-fast, gram-positive bacteria that are responsible for diseases causing public health problems worldwide (2, 5, 33). The increased concern about infections caused by *Mycobacterium tuberculosis*, coupled with the emergence of life-threatening infections caused by *Mycobacterium avium* and *Mycobacterium intracellulare* in human immunodeficiency virus-infected individuals, dictates that the mechanisms of gene regulation in these bacteria be identified. An understanding of the regulatory sequences involved in the expression of virulence determinants in pathogenic mycobacteria may aid in the development of new vaccines and more effective medical treatments.

Presently, very little is known about the genetic elements that contribute to the control of gene expression in mycobacteria. Mycobacteria have G+C-rich genomes. It is known that in bacteria, the overall G+C content of the genome affects codon usage, the choice of translation initiation and termination codons, and the promoter recognition sites for RNA polymerase (27, 29). It is known that in prokaryotic promoters, σ-factor components of RNA polymerase holoenzymes interact with the DNA sequences located approximately 35 and 10 bp upstream from the start site of transcription. These interactions are critical in determining the timing and levels of gene expression (10, 25). Recently, several mycobacterial promoters have been identified (19, 20, 26, 28, 37, 38, 42, 44, 46) and their start points of transcription have been mapped, but only one promoter has been subjected to genetic analysis to identify the nucleotides important in determining promoter function (28). A few of the analyzed mycobacterial promoters appear to resemble the typical *Escherichia coli* σ^{70} consensus promoter, but most have a G+C content ranging between 65 and 70% and differ from the *E. coli* consensus.

We have examined the structure of the promoter of the *rpsL* gene of *Mycobacterium smegmatis*, which encodes the ribosomal protein S12. We chose the *rpsL* gene because in other bacterial systems this gene is highly expressed (31). We chose to work with *M. smegmatis* because it is fast growing, nonpathogenic, and more easily transformable than other mycobacterial species (13). The *M. smegmatis rpsL* and *rpsG* genes, encoding the ribosomal proteins S12 and S7, have been cloned, and their DNA sequences have been determined (17). The structural arrangement of these genes is identical to that seen in *E. coli* and other eubacteria, but the genes have an overall G+C content (65%) and codon usage typical of mycobacteria (7, 47). The codons are 85% G+C in the third position. The gene for ribosomal protein S12 precedes the gene for ribosomal protein S7 and the coding regions of these two genes overlap, suggesting that they are part of an operon.

We identified the start site for transcription initiation of the *rpsL* gene, and by constructing *rpsL-xylE* transcriptional fusions (6, 18), we used deletion analysis to delineate the sequences critical for promoter activity and examined the effects of base substitutions on utilization of the promoter in vivo.

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TABLE 1. Oligonucleotides used in this study[a]

Oligonucleotide	DNA sequence (5′-3′)	Mutation(s)
N1	TCGAGCTAGCCCAACCAGCCCGCGAAC	
N2	TCGAGCTAGCAACCACACGAAACAT	
R1	GCGAAACGCCGAGTTCCCGTGCAACG	
R2	CGTTGCACGGGAACTCGGCGTTTCGG	
T1	AATTAGCCCGCCTAATGAGCGGGCTTTTTTTTG	
T2	AATTCAAAAAAAAGCCCGCTCATTAGGCGGGCT	
B1	TGCAGGATCCGGCTTTCTCTGTGTTGC	
E1	TCGAGAATTCACCGATATGCTGCTG	
5′1	TCGAGAATTCTCTAGAGTCGAG	
5′2	TCGAGAATTCCGGGATGGAGCGGTC	
5′3	TCGAGAATTCGCGAACGTAGGGTGG	
5′4	TCGAGAATTCTGACCTGCGATTAGAC	
5′5	TCGAGAATTCGTCGCGGTCGGCCC	
5′6	TCGAGAATTCGTGCCTGGCCCGAAAG	
5′7	TAGCGAATTCGCATGTCTATCACGC	
3′1	TCGAGGATCCGCGACGAAACAAACTCG	
3′2	TCGAGGATCCCGGCGTGCGGGCACGCT	
PCR1	GCGAATTGGCCCGGCGTGCGGGCAC	
PCR2	GGTCGCATTACACCTTTGTTCATGACG	
M1	GACGAAACAGACTCGTGCAC	−38T→C
M2	GACGAAACGAACTCGTGCAC	−37T→C
M3	GCGACGAGACAAACTCGTGC	−34T→C
M4	GATCCACCACGATACCAGG	−11T→C
M5	GATCCACCAGAATACCAGG	−10G→C
M6	GATCCACCGCAATACCAG	−9T→C
M7	GCACGATCCGCCACAATACC	−6T→C
M8	GATCCACCACAATAGAGGGGCCGACCGCGA	−17T→C, −16G→T, −15G→C
MUT1	AATTAGCCCGCCTAATGAGCGGGCTTTTTTTTG	
MUT2	GGTCGCATTACACCTTTGTTCATGACG	
XYLE2	GTCCAGTACACGCAGCTGCACAT	
PE1	CTGCGGCTGCCCTTGA	
TBRPS	CGACCCTTGCGGACCAGCTGCTGG	
RPSL2	GGCCTAGCCCCGCCACGGTTACCC	
RPSL3	GGAACATGCGAATTGGCCCGCATC	
RPSL4	GCTGTGGGTTGCCCTTCAGAGCCGC	

[a] For oligonucleotides used in directed mutagenesis, the base that differs from the wild-type sequence is underlined and the position, wild-type base, and mutation in the *rpsL* promoter region are indicated.

MATERIALS AND METHODS

Bacterial strains. *M. smegmatis* LR222 was provided by Thomas Shinnick, Centers for Disease Control.

General cloning procedures. Restriction endonuclease cleavage reactions and ligations were done according to the manufacturers' specifications (Boehringer Mannheim Biochemicals and New England Biolabs). DNA fragments used for cloning and labeling reactions were purified by electrophoresis on low-gelling-point agarose (FMC). After ligation, recombinant plasmids were transformed into *E. coli* DH5α (35) and bacteriophage M13 recombinants were transfected into *E. coli* 71.18 (49). Plasmid transformants were selected by growth on Luria broth (LB) plates containing 50 μg of kanamycin per ml. M13 recombinants were identified as white plaques on plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Plasmid derivatives of pTKmx in *M. smegmatis* were selected and maintained on LB agar plates containing 50 μg of kanamycin per ml and 0.05% (vol/vol) Tween 80.

DNA sequencing. Fragments were cloned into M13mp18 and M13mp19 phages and sequenced by a dideoxy sequencing protocol with Sequenase (United States Biochemicals) (36, 43) by using the dGTP analogs dITP and 7-deaza-dGTP to resolve compression artifacts.

Plasmid construction. Plasmid pTKmx was derived from pxylE1 (39). The mycobacterial replicon from pJC85, a pAL5000 derivative (23), was isolated by overlapping PCR gene construction (recombinant PCR) (45) with the oligonucleotides N1, N2, R1, and R2 (Table 1). Primers N1 and R2 and, in a separate reaction, primers N2 and R1 were used to amplify a 1,290-bp fragment and a 1,050-bp fragment, respectively. These DNA fragments were purified, combined, denatured, and then annealed, and this product served as a template for a PCR using the primers N1 and N2. The N1 and N2 primers created *Nhe*I restriction sites on the ends of the resulting 2,340-bp fragment, and the R1 and R2 primers were used to remove an internal *Eco*RI site within the replicon. The resulting 2,340-bp fragment was digested with *Nhe*I and ligated into vector pxylE1 (39) to generate pTKx. To eliminate read-through transcription into the promoterless *xylE* gene, two oligonucleotides, T1 and T2 (Table 1), were annealed to create a 33-bp fragment containing a *trpA* transcription terminator, which was inserted at the unique *Eco*RI site of pTKx, recreating an *Eco*RI site at the 3′ end of the terminator. A second copy of the *trpA* terminator was inserted in an identical manner to create the final plasmid, pTKmx (Fig. 1).

PCR. PCRs were performed according to the Taq polymerase manufacturer's specifications (Perkin-Elmer), except that dimethyl sulfoxide was added to a final concentration of 5%. Each of the 30 cycles was done at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and the reaction was finished with an incubation of 10 min at 72°C.

Fragment purification. PCR products were purified by using Promega's Wizard PCR Preps DNA Purification System and then isolated from low-gelling-point agarose. Gel slices were heated to 65°C for 5 min, and heating was followed by three phenol extractions, one chloroform extraction, and precipitation with 2 volumes of ethanol.

Construction of deletion derivatives. All DNA fragments containing deletion derivatives of the *rpsL* promoter were synthesized by PCR amplification. Oligonucleotides (Table 1) were designed to generate either an *Eco*RI or a *Bam*HI site at the ends of the fragments. The DNA template in each case was a pUC19 derivative with a 1,600-bp *Sal*I fragment that included 400 bp of DNA upstream of the *rpsL* coding region, as well as the entire *rpsL* gene (17). The DNA fragments were generated with the following primers: 360-bp fragment, primers 5′1 and B1; 333-bp fragment, primers E1 and B1; 306-bp fragment, primers 5′2 and B1; 293-bp fragment, primers 5′3 and B1; 267-bp fragment, primers 5′4 and B1; 231-bp fragment, primers 5′5 and B1; 200-bp fragment, primers 5′6 and B1; 133-bp fragment, primers 5′7 and B1; 114-bp fragment, primers E1 and 3′1; 97-bp fragment, primers 5′5 and 3′2.

Cycle PCR sequencing. The nucleotide sequence of purified PCR fragments was determined by dideoxy sequencing by using the Promega fmole cycle sequencing kit with oligonucleotide primers PCR1 and PCR2 (Table 1), internal to the *rpsL* promoter and complementary to a region at the 5′ end of the *xylE* gene, respectively. The reaction mixtures were incubated for 30 cycles of 94°C for 30 s and either 68 or 70°C for 30 s.

Oligonucleotide-directed mutagenesis. The sequences of the eight mutagenic oligonucleotides M1 to M8 are shown in Table 1. Oligonucleotides M1 to M7

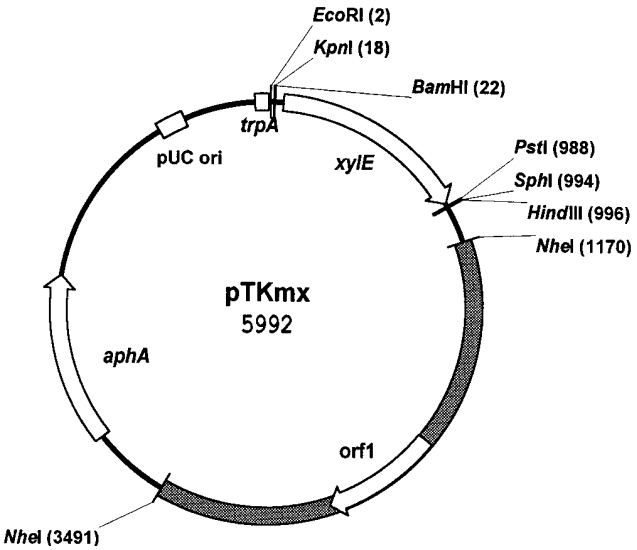

FIG. 1. Map of pTKmx. The cross-hatched box represents mycobacterial pAL5000 DNA containing the *orf1* reading frame essential for replication (23). The box labeled *trpA* represents two tandem copies of the 28-bp *trpA* transcription terminator. pUC ori denotes the pUC18 origin of replication, *aphA* is the kanamycin resistance gene from Tn*5* (1), and *xylE* is a promoterless *xylE* reporter gene. The direction of transcription of the different genes is indicated by arrows.

were used to mutagenize the 267-bp *Eco*RI-*Bam*HI DNA fragment from pTKmx.267 that contained the *rpsL* promoter, which was cloned between the *Eco*RI and *Bam*HI sites of M13mp18 DNA, by the procedure of Kunkel (22). Oligonucleotide M8 was used to mutagenize the −38T→C (T-to-C change at position −38), the −37T→C, and wild-type versions of the 267-bp *rpsL* promoter fragment in an identical manner. The wild-type and the mutagenized *rpsL* promoters were then digested with *Eco*RI and *Bam*HI and cloned next to the promoterless *xylE* gene in pTKmx.

PCR mutagenesis. The 267-bp *Eco*RI-*Bam*HI DNA fragment containing the *rpsL* promoter was amplified from pTKmx.267 for 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The primers used for the amplification were Mut1 and Mut2 (Table 1), which are external to the *Eco*RI-*Bam*HI fragment. PCR mutagenesis mixtures included 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, and 0.2 mM dGTP (3). Amplified fragments were digested with *Eco*RI and *Bam*HI, cloned as a mixed population into pTKmx, and transformed into *E. coli* DH5α. The resulting transformants were pooled, grown in 500 ml of LB containing 50 μg of kanamycin per ml, and subjected to an alkaline lysis plasmid procedure (35). The resulting mixed population containing mutant plasmids was electroporated into *M. smegmatis* LR222, and the transformants were screened for the loss of *xylE* activity by spraying the plates with a 0.5 M catechol solution.

RNA isolation. RNA was purified by centrifugation through CsCl by a modification of the procedure of McDonald et al. (24). *M. smegmatis* strains were grown in 200 ml of LB containing 0.05% (vol/vol) Tween 80 to an optical density at 550 nm (OD_{550}) of 0.8. The cells were pelleted at 6,000 × *g* for 10 min, and the pellet was suspended in 3.5 ml of guanidine isothiocyanate buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate [pH 5.0], 0.05% sarcosyl, and 1 M 2-mercaptoethanol). The cells were broken by passage twice through a French pressure cell at 20,000 lb/in^2. The lysate was then layered on a cushion of 1.5 ml of 5.7 M CsCl containing 25 mM sodium acetate (pH 5.0) in 5-ml polyallomer tubes. The tubes were centrifuged at 32,000 rpm at 18°C for 16 h in a Beckman SW 50.1 rotor. The RNA pellet was suspended in Tris-EDTA (TE) buffer (pH 8.0) and extracted twice with phenol-chloroform, with 1% (vol/vol) diethylpyrocarbonate in the first extraction. The RNA was precipitated twice with 2 volumes of ethanol, and the final pellet was dissolved in 200 μl of TE.

S1 nuclease mapping. S1 mapping experiments were done as described elsewhere (48), with the following exception. RNA (20 μg) was mixed with 0.2 μg of ^{32}P-5′-end-labeled DNA and precipitated with ethanol. To make the labeled DNA, 100 ng of an oligonucleotide, xylE2 (Table 1), complementary to a region at the 5′ end of *xylE*, was labeled with [γ-^{32}P]ATP and annealed to 18 μg of plasmid pTKmx.360 which had been linearized with *Eco*RI. The primer was extended with the Klenow fragment of DNA polymerase I for 30 min at 37°C. The single-stranded labeled fragment was purified on an alkaline agarose gel following autoradiography, as described elsewhere (8).

Primer extension analysis. A radiolabeled oligonucleotide primer, PE1 (Table 1), that was complementary to a region downstream from the *rpsL* promoter was allowed to hybridize with LR222 RNA isolated during exponential growth. Hybridization conditions were 1 min at 90°C, 10 min at 65°C, and 15 min on ice. After hybridization, the primer was extended with reverse transcriptase (42°C, 1 h). The same oligonucleotide was used to prime dideoxy-sequencing products from a DNA template that contained the *rpsL* gene to generate a sequence ladder.

Electroporation of *M. smegmatis*. Two-hundred-milliliter cultures of *M. smegmatis* were grown in LB supplemented with 0.5% Tween 80 to an OD_{550} of 0.8. The culture was centrifuged at 6,000 × *g* for 10 min, and the pellet was suspended in 100 ml of ice-cold 10% glycerol and centrifuged for 10 min. The pellets were then washed three times in a volume of 50 ml of ice-cold 10% glycerol, suspended in 2 ml of 10% glycerol, and quickly frozen in a dry-ice ethanol bath. Electroporation conditions were 1,000 Ω, 25 μF, and 2,500 V (15). Time constants averaged 18 ms.

Assay of catechol 2,3-dioxygenase activity. Mycobacterial colonies on LB plates supplemented with 0.05% (vol/vol) Tween 80 were sprayed with a solution of 0.5 M catechol. For quantitative liquid assays, 10-ml samples of cells were centrifuged, washed with 20 mM potassium phosphate buffer (pH 7.2), and centrifuged again, and the pellets were stored overnight at −80°C. The pellets were suspended in 2 ml of APEL buffer (100 mM potassium phosphate [pH 7.5], 20 mM EDTA, 10% acetone, 1 mg of lysozyme per ml) and incubated for 15 min at 37°C. The cells were lysed by two 30-s sonication pulses, and then 20 μl of 10% Triton X-100 was added and the cell extracts were placed on ice for 15 min. Cell debris was removed by centrifugation. Catechol 2,3-dioxygenase specific activities were determined spectrophotometrically at 30°C in the presence of 0.2 mM catechol at OD_{375}. Protein concentrations were measured by the Bio-Rad assay. One milliunit corresponds to the formation of 1 nmol of 2-hydroxymuconic semialdehyde per min at 30°C (14, 18).

Chromosomal sequencing of the *M. tuberculosis* (H37Rv) *rpsL* promoter region. Four oligonucleotides were used to directly sequence chromosomal DNA from *M. tuberculosis* H37Rv: TBRPS, RPSL2, RPSL3, and RPSL4 (Table 1). Oligonucleotides were labeled at the 5′ end with [γ-^{32}P]ATP and used in a dideoxy DNA cycle sequencing reaction (Promega fmol DNA sequencing system) under the following conditions: 95°C for 2 min and then 30 cycles of 95°C for 30 s and 72°C for 30 s.

RESULTS

Mapping the 5′ end of the *rpsL* transcript. We have previously reported the nucleotide sequence of the *rpsL* gene of *M. smegmatis* (17). To map the start point of transcription of the *rpsL* gene, we performed a primer extension experiment (Fig. 2A). RNA was extracted from exponentially growing LR222 cells. A radiolabeled oligonucleotide that was complementary to a region within the *rpsL* gene was hybridized to the RNA, and the primer was extended by using reverse transcriptase. The size of the DNA copy of this mRNA was identified by comparing its electrophoretic mobility with that of a DNA sequence ladder generated by annealing the same oligonucleotide primer to a plasmid carrying the *rpsL* DNA. The major transcription start point mapped to a G residue, on the nontranscribed strand, 199 bp upstream from the *rpsL* translation initiation codon (Fig. 2A). A faint band was seen approximately 80 bp further upstream and may indicate the presence of a second, weaker promoter (Fig. 2A) (see below). In addition, a strong band was seen 41 bp upstream from the start point of translation (Fig. 2A). This band was presumably due to premature termination of synthesis by reverse transcriptase because analysis of transcriptional gene fusions described below rules out the possibility that this band represents a transcription start point.

The *rpsL* promoter was then cloned as a 267-bp *Eco*RI-*Bam*HI fragment into plasmid pTKmx (Fig. 1), between its unique *Eco*RI and *Bam*HI restriction enzyme cleavage sites, to create plasmid pTKmx.267. Transformation of this plasmid into LR222 resulted in colonies that became bright yellow when plates were sprayed with an aqueous solution of catechol, indicating that the *rpsL* promoter was driving expression of the promoterless *xylE* gene carried by pTKmx. To determine if the *rpsL* promoter in plasmid pTKmx.267 initiated transcription from the same site as the chromosomal copy of the *rpsL* gene, the 5′ termini of the RNA transcript that was encoded by the plasmid was mapped by S1 nuclease protection experiments (Fig. 2B). A single-stranded DNA probe that was labeled at its

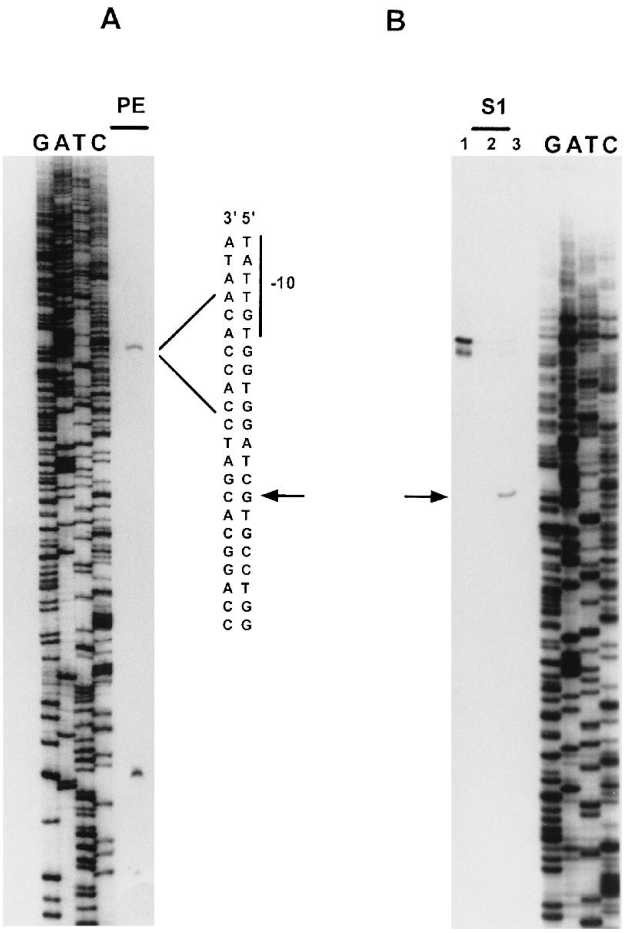

FIG. 2. Mapping the transcription start site of the *rpsL* gene. (A) Primer extension analysis. The first four lanes, labeled G, A, T, and C, show a DNA sequence ladder of the *rpsL* promoter region. The fifth lane, labeled PE, shows the results of primer extension analysis with the same oligonucleotide used to generate the sequence ladder. The major extension product is indicated, and the G residue on the nontranscribed strand where transcription initiates is shown by the arrow. (B) S1 nuclease protection analysis. Lane 1, probe alone; lane 2, LR222 RNA containing no *xylE* plasmid; lane 3, LR222 harboring the pTKmx.267 plasmid. The S1-resistant RNA in lane 3 is indicated with an arrow. Lanes G, A, T, and C show the sequence ladder generated with the same oligonucleotide used to synthesize the probe.

5′ end and extended from within the *xylE* coding sequence through the *rpsL* promoter sequence was made. RNA was isolated from exponentially growing LR222 cells that contained the pTKmx.267 plasmid and annealed to the probe. Following treatment of RNA-DNA hybrids with S1 nuclease, S1 nuclease-resistant DNA was compared by electrophoresis with a DNA sequence ladder generated by the same oligonucleotide used to produce the single-stranded DNA probe (48). The RNA from the strain containing the plasmid bearing the *rpsL-xylE* transcriptional fusion protected the DNA probe from digestion with S1 nuclease (Fig. 2B, lane 3), but the RNA from the parent strain with no *rpsL-xylE* fusion did not (lane 2). The 5′ end of the transcript produced from the plasmid-borne *rpsL-xylE* fusion was identical to the one originating from the chromosomal copy of the *rpsL* gene, 199 bp upstream from the translation initiation codon.

Deletion analysis of the *rpsL* promoter. In order to determine which of the upstream sequences were essential for *rpsL* promoter function, deletion derivatives of the promoter were generated. PCR amplification was used to create DNA fragments with convenient restriction sites for subsequent cloning steps. The nucleotide sequence of individual cloned promoter fragments was determined to ensure that no mutations were caused by the amplification reaction. These various promoter fragments were fused to the *xylE* reporter gene in plasmid pTKmx (Fig. 3). Plasmid pTKmx.267 contained sequences that began at the translation start codon and extended 267 bp upstream of the translation initiation. It produced high levels of *xylE* activity (>1,200 mU/mg) and was completely stable during growth in liquid or on solid media. All of the plasmids that carried promoter fragments containing DNA sequences starting at the translation initiation site and extending more than 267 bp upstream (pTXmx.293 to pTKmx.360) were structurally unstable after several generations of growth, spawning colonies that had lost *xylE* activity and contained plasmids bearing deletions of the *rpsL* promoter region. This instability was presumably due to the expression of *xylE*, because when these longer promoter fragments were cloned into a shuttle plasmid lacking the *xylE* gene they were completely stable. Plasmid pTKmx.231 expressed levels of *xylE* activity similar to those of pTKmx.267, but plasmids pTKmx.200 and pTKmx.133, which contained sequences that originated at the translation initiation site and extended 200 and 133 bp upstream, respectively, had no activity upon transformation into LR222. These results indicated that sequences essential for *rpsL* promoter function and for the production of a 5′ transcript end detected in primer extension and S1 nuclease protection experiments lay between nucleotides 200 and 231 upstream of the translation initiation codon. Furthermore, the strong band corresponding to a 5′ transcript end 41 bp upstream from the translation start that was seen in the primer extension experiment (Fig. 2A) was presumably due to premature termination of the reverse transcriptase and not to the

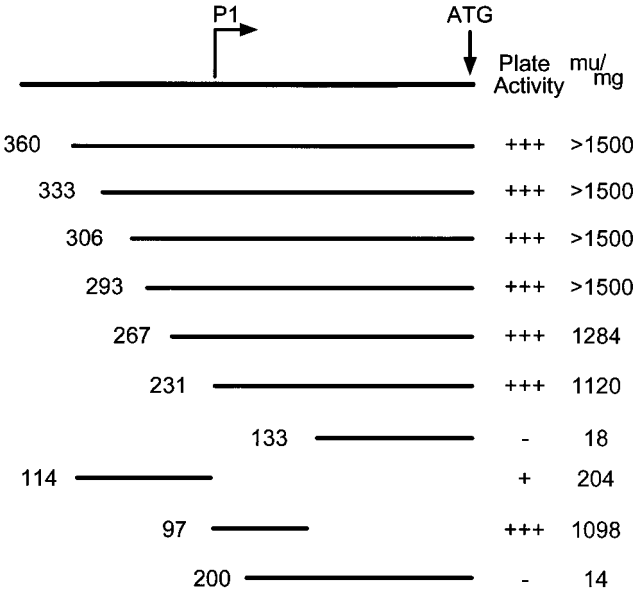

FIG. 3. Deletion derivatives of the *rpsL* promoter region fused to the *xylE* reporter gene. The start site for transcription initiation is indicated by the arrow labeled P1. The location of the ATG translation initiation codon is marked. Plate activity refers to the color change that occurred when colonies containing the fusions were sprayed with an aqueous solution of catechol: +++, bright yellow; +, pale yellow; −, white. The catechol 2,3-dioxygenase specific activity of mid-exponential-phase cultures is shown in milliunits per milligram. The results are the average for three experiments.

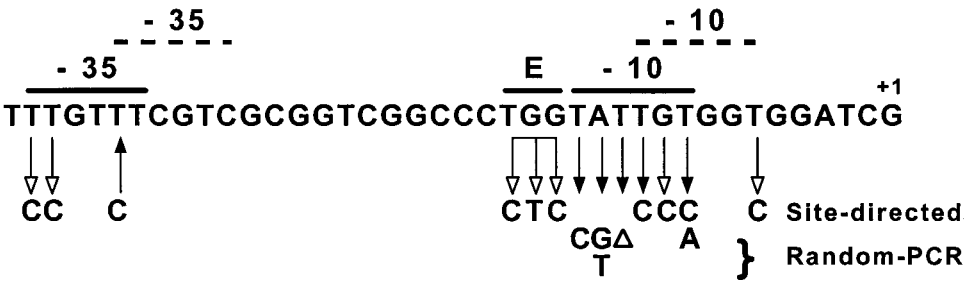

FIG. 4. Nucleotide sequence of the *rpsL* promoter of *M. smegmatis*. The nucleotide sequence of the nontranscribed strand of the *rpsL* promoter is shown. Transcription is initiated at the G residue indicated by +1. Hexameric −35 and −10 consensus sequences defined in this paper are indicated. Putative −35 and −10 regions discussed in the text are indicated by a dashed bar. Nucleotide substitutions are indicated below the arrow. The base substitutions in the top line were created by site-directed oligonucleotide mutagenesis. The base substitutions in the bottom two lines were found following random PCR mutagenesis. Mutations with little or no effect on promoter function are indicated by open arrowheads. Mutations with severe effects are indicated by filled arrowheads. An up arrow indicates a small increase in promoter activity. The location of the 3-bp change in the extended −10 region is indicated by the bar labeled E.

presence of a promoter. These results were confirmed by constructing the plasmid pTKmx.97, which carried a segment of *rpsL* DNA extending from 133 to 231 bp upstream of the translation initiation codon. This plasmid produced levels of *xylE* activity similar to those produced by plasmids pTKmx.231 and pTKmx.267.

Plasmid pTKmx.114 contained DNA sequences that started 231 bp from the start point of translation and extended 114 bp further upstream (Fig. 3). This plasmid produced significant levels of *xylE* activity. Coupled with the faint band corresponding to a 5′ transcript end 280 bp upstream of the translation start observed in primer extension experiments, this indicated that a second, less active promoter might be present further upstream of the major promoter. This second promoter might contribute to the instability of the longer promoter fragments.

Isolation and characterization of *rpsL* promoter mutants. The nucleotide sequence immediately upstream from the start point of transcription of the *rpsL* promoter contains four sequences that are similar to the canonical sequences recognized by *E. coli* σ^{70} (25) (Fig. 4). There are two potential −10 hexamers 6 and 9 bp upstream of the transcription start point. The −10 hexamer 6 bp upstream matches the typical *E. coli* σ^{70} consensus hexamer at three of six positions, while the −10 hexamer 9 bp upstream matches the *E. coli* σ^{70} consensus at four of six positions. There are also two potential −35 hexamers. One is located 17 bp from the −10 hexamer 6 bp upstream of the transcription start point and matches the *E. coli* σ^{70} consensus hexamer in two of six positions. The second is located 18 bp from the −10 hexamer 9 bp upstream of the start point of transcription and matches the *E. coli* σ^{70} consensus hexamer at three of six positions.

To determine which base pairs in the *rpsL* promoter were critical for promoter activity, we used oligonucleotide-directed mutagenesis to construct single nucleotide substitutions in the 267-bp *rpsL* promoter fragment carried by pTKmx.267 (22). We changed conserved positions in the −10 region (positions −6, −9, −10, and −11) and in the −35 region (positions −34, −37, and −38). All of the changes were to a CG base pair. The resulting mutant promoter fragments were then fused to *xylE* in plasmid pTKmx and assayed for their ability to direct the transcription of the *xylE* gene in vivo. If these nucleotides constituted part of a recognition site for RNA polymerase, it would be expected that they would severely affect promoter function. Only one change in these conserved residues (−9T→C) abolished promoter activity. The T→C change at position −11 resulted in 50% of wild-type promoter activity. None of the changes in the −35 region had a detrimental affect on promoter activity. Furthermore, the −34T→C change increased promoter activity by 10%. Since the T→C change at position −6 did not alter promoter function and mutations at positions −9 and −11 did have a negative effect, it seemed likely that the −10 region 9 bp upstream of the transcription start site was recognized in vivo. We also concluded from these experiments that the *xylE* reporter gene system was sufficiently sensitive and reproducible that we could use it to screen for random, rather than targeted, mutations.

Random PCR mutagenesis (3) was used to create an unbiased set of promoter mutations. To determine the nature of the mutations conferring loss of promoter activity, total DNA was isolated from each mutant strain, amplified by PCR (34) using oligonucleotide primers external to the promoter sequence of the *rpsL* gene, and sequenced directly with a single internal primer in a linear amplification reaction (double-strand cycle sequencing). Only promoters that contained a single nucleotide substitution were chosen for further study (Fig. 4). A total of five different independent mutations that abolished promoter activity in vivo were found, and most of these mutants were isolated several times, indicating that a complete screen of potential mutants was achieved. All of the mutations that severely reduced the amount of *xylE* produced from the promoter mapped to the −10 region 9 bp upstream of the transcription start site. A T→C transition at position −14, a substitution of either a G or a T at position −13, a deletion of a T at position −12 (or position −11), and a T→A transversion at position −9 completely eliminated promoter activity (Fig. 5A). The position of these mutations that reduced or abolished promoter function, as well as our inability to isolate mutants with deleterious mutations at other positions in the −10 region, showed that the −10 region 9 bp upstream of the transcription start site was essential for promoter function. The distribution of mutations in the −10 region that affected promoter activity closely paralleled the effects of nucleotide substitutions that reduce activity in the −10 hexamer recognized by *E. coli* σ^{70} (25).

We did not find any mutations that reduced promoter activity in the −35 region. Furthermore, plasmid pTKmx.231 did not carry the −35 hexamer 18 bp upstream of the −10 region essential for promoter function yet expressed almost the same level of *xylE* activity as did pTKmx.267, which carried the entire −35 region. These results suggested that the −35 region was not required for *rpsL* promoter function. Several promoters that do not require a −35 region for activity have been studied (4, 16, 21, 30). The motif TGn is characteristically found immediately upstream from the −10 region hexamer in these promoters, producing what is described as an extended −10 region. The nucleotides TGG are found in this position in the *rpsL* promoter. To determine if these bases played a role in promoter activity, oligonucleotide-directed mutagenesis was

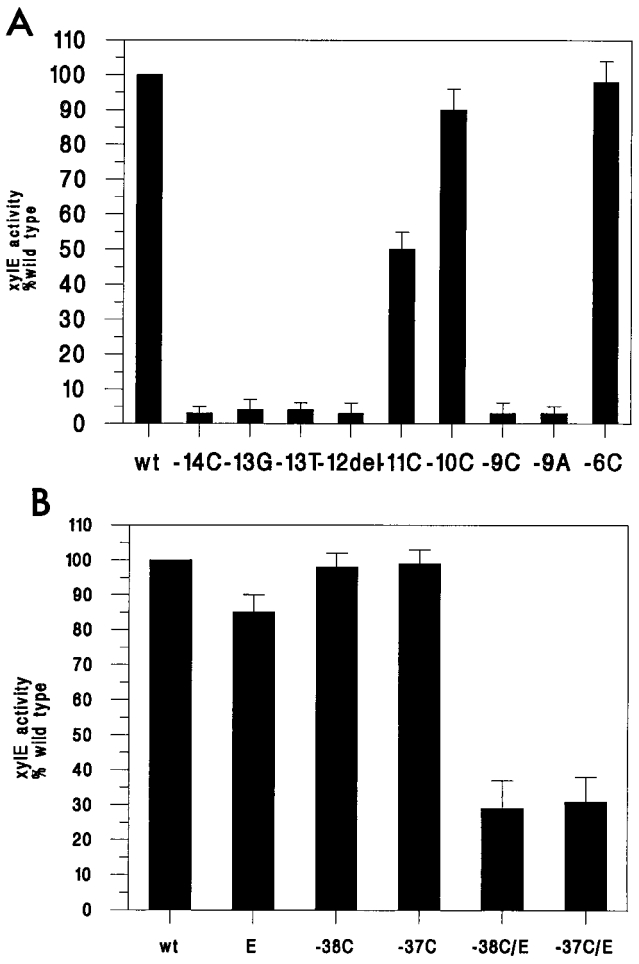

FIG. 5. Quantitative data from *xylE* assays. Levels are expressed as a percentage of the maximum activity found in the wild-type promoter and are the average for three experiments. The range in values is indicated with error bars. The mutant alleles are marked below the bars and are numbered relative to the transcription start site. (A) Effects of oligonucleotide site-directed mutations and random PCR mutations. (B) Effects of mutations in the extended −10 region and −35 region. wt, wild type.

used to simultaneously change the TGG nucleotides at positions −17, −16, and −15 to CTC. This triple mutation (named E, for "extended") was created in the wild-type promoter and in two mutant derivatives of the promoter containing mutations in the −35 region. When the mutant promoter containing only the E substitutions was fused to *xylE*, a 15% reduction in promoter activity was seen (Fig. 5B). However, a combination of the E substitutions and a T→C change at either position −38 or position −37 reduced promoter activity by 70% relative to wild-type levels. These results demonstrated that in the absence of a functional −35 region, the nucleotides in the extended −10 region are essential for normal promoter activity and that in the absence of this extended −10 motif, the −35 region 18 bases upstream of the essential −10 hexamer was required for normal promoter function.

Comparison of the *rpsL* promoter region with homologous regions from other mycobacteria. The nucleotide sequence of the homologous region of *M. tuberculosis* was determined by PCR cycle sequencing using chromosomal DNA isolated from strain H37Rv as the template. Comparison of homologous DNA sequences from *Mycobacterium leprae*, *Mycobacterium bovis*, *M. tuberculosis*, and *M. smegmatis* is shown in Fig. 6. The untranslated leader mRNA sequence was only 45% conserved between *M. smegmatis* and the slowly growing mycobacterial species. However, the putative −35 and −10 hexameric recognition sequences for transcription were 75% conserved among the three species, including absolute conservation in the −10 hexamer. There was also sequence conservation around the start point of translation, as well as a 20-bp sequence about 140 bp upstream from the translation start site which was highly conserved among the four species. The significance of this conserved region is currently unknown. Only the promoter of *M. smegmatis* contained the extended −10 TGn motif.

DISCUSSION

Identification of the *rpsL* promoter. We have used a combination of biochemical and transcriptional gene fusion techniques to identify the start point of transcription of the *rpsL* gene 199 bp upstream from the start point of translation. A long untranslated leader region upstream from the *rpsL* gene has been found in several other bacteria, including *Micrococcus luteus* (27).

The G residue where transcription started in the *rpsL* promoter was preceded by a C at position −1 and followed by at T at position +2, and those are the most common nucleotides found at positions −1 and +2 in *E. coli* promoters (10, 11). Transcription initiated 8 bp downstream from the −10 hexamer defined by site-directed and random mutagenesis (Fig. 4). The *rpsL* promoter of *M. smegmatis* had a somewhat surprising structure for a G+C-rich organism, being identical to the canonical *E. coli* σ^{70} consensus promoter at four of six positions in the −10 hexamer and at three of six positions in the −35 hexamer, which was 18 bp upstream of the −10 hexamer (Fig. 4). The DNA sequences in the *rpsL* promoter that corresponded to the *E. coli* consensus are the nucleotides that have been shown to be the most conserved in the canonical *E. coli* promoter (25). In a study of randomly cloned *M. smegmatis* promoter fragments fused to a chloramphenicol acetyltransferase reporter gene, only 12% of the promoters that were active in *M. smegmatis* had activity in *E. coli* (9). This suggests that the G+C-rich nature of the mycobacterial genome may result in the presence of promoters that differ substantially from canonical promoters in other bacteria. Analysis of a promoter region in mycobacteriophage L5 shows the presence of three promoters, two of which are similar to *E. coli* promoters and one of which is quite different (28). Recently, a compilation of 139 promoters from strains of *Streptomyces*, a genus with a G+C content that ranges between 69 and 78%, showed that 21% of the promoters analyzed resembled the typical *E. coli* σ^{70} promoter, and most of these directed the transcription of housekeeping genes (41). Therefore, it seems probable that a similar situation exists in mycobacteria and that mycobacterial ribosomal promoters will resemble *E. coli* σ^{70} promoters. One would also assume that because ribosomal genes are heavily transcribed, these promoters are recognized by the major form of RNA polymerase in mycobacteria. This mycobacterial RNA polymerase has been shown to have marked conservation with that of *E. coli* (32).

Mutational analysis of the *rpsL* promoter. All of the mutations that abolished promoter activity were changes away from the conserved nucleotides that matched the *E. coli* σ^{70} canonical −10 hexamer. No mutations in the −35 region that reduced or eliminated promoter activity were found. A deletion of the promoter fused to *xylE* (pTKmx.231) that removed the −35 region and upstream sequences maintained almost 90% of the wild-type promoter activity. Since fragments of DNA cloned into pTKmx are buffered from transcription originating

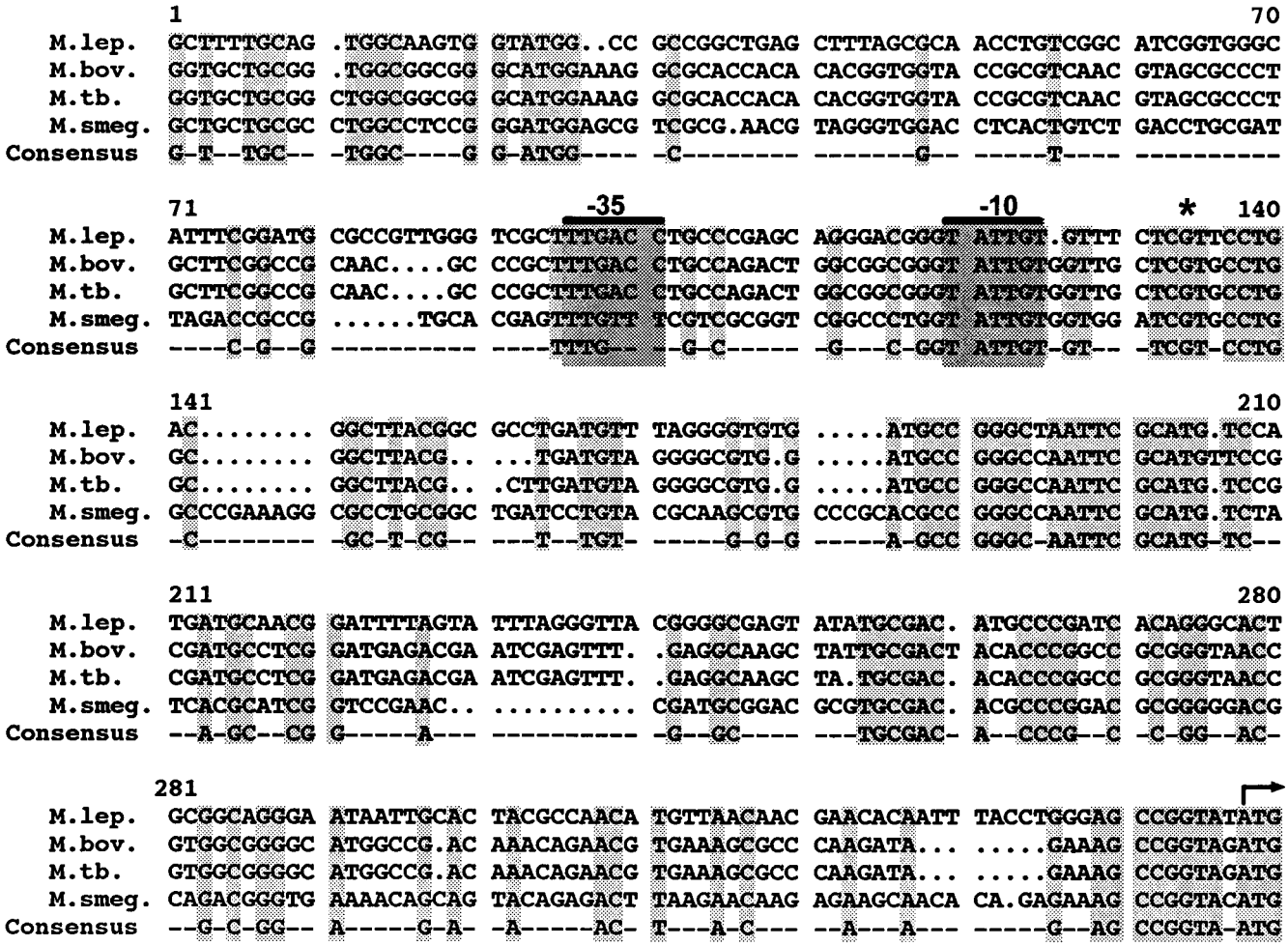

FIG. 6. Alignment of mycobacterial promoter regions upstream from the *rpsL* (S12 protein) translation start site. The nucleotide sequences upstream from the *rpsL* coding sequences of *M. smegmatis* (M. smeg.), *M. leprae* (M. lep.) (12), *M. bovis* (M. bov.) (38a), and *M. tuberculosis* (M. tb.) are shown. The consensus sequence is indicated by shading when the nucleotide is conserved among all four species. The translation initiation codon is indicated by an arrow, and the location of the start point of transcription for the *M. smegmatis rpsL* gene is shown with an asterisk. The putative −35 and −10 hexamers are indicated by the darker shading.

from plasmid sequences by tandem *E. coli trpA* transcription terminator sequences, this showed that this region was not essential for promoter function. These results showed that the product observed in primer extension and nuclease protection experiments did not arise by processing of a longer transcript and that the conserved −10 hexamer was essential for promoter function. Furthermore, they suggested the possibility that the *M. smegmatis rpsL* promoter contained an extended −10 region.

Extended −10 regions occur naturally in approximately 16% of *E. coli* promoters (10, 21). The extended −10 site is a TGn motif immediately upstream of the −10 hexamer; this motif is found in association with different versions of a −35 hexamer. In the absence of a functional −35 region in *E. coli*, the activity of the galactose operon promoters P1 and P2 in a Δ*crp* background is dependent on the extended −10 region (4, 30). Nucleotide changes at position −14 in either of these promoters eliminate promoter activity. This activity can be restored if a functional −35 region is restored (4, 30). It has been proposed that in the absence of a functional −35 region, the extended −10 region functions in binding of RNA polymerase to the promoter as well as in subsequent steps of transcription initiation (4, 16, 21, 30). The additional DNA-protein contact in the extended −10 region is thought to play a role in open complex formation. Our mutational analysis indicated that in the absence of a functional −35 region, the extended −10 region was essential for normal promoter activity, perhaps by providing additional contacts on the DNA for RNA polymerase. It is likely that the mutations in the extended −10 region eliminated certain contact sites that are used by RNA polymerase, and in the absence of a functional −35 region these contacts are critical for promoter function.

Significance of the extended −10 motif. There are two other mycobacterial promoters that show an extended −10 motif, the *M. bovis hsp60* P2 promoter (40) and the *M. leprae* 16S rRNA promoter (37). The three slowly growing mycobacteria, *M. leprae*, *M. tuberculosis*, and *M. bovis*, do not have an extended −10 motif in the *rpsL* promoter, but they do possess a 5- of 6-bp match with the −35 *E. coli* consensus hexamer. Ribosomal promoters are transcribed more frequently than other promoters; therefore, RNA polymerase must be able to interact efficiently with these promoters. The differences in structure of mycobacterial *rpsL* promoters may reflect differences between the primary sigma factors of fast and slowly growing mycobacteria, so that sigma factors make contact with the −10 region and additional upstream sequences, either in the −35 region or in the extended −10 motif, or these primary sigma factors may function in a similar way and are capable of initi-

ating transcription at promoters with both −35 regions and extended −10 regions.

ACKNOWLEDGMENTS

We thank Thomas Shinnick and Daniel Stein for the gifts of bacteria and plasmid DNA. H37Rv DNA was provided by John T. Belisle. We also thank our colleagues, especially Charles P. Moran, Jr., and Adriano Henriques for helpful comments and suggestions.

This work was funded by grant AI35285 from NIH and a fellowship from the Heiser Foundation to T.K. H37Rv DNA was produced through funds from NIH contract NO1 AI25147, "Tuberculosis Research Materials."

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