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Identification and Characterization of the *dps* Promoter of *Mycobacterium smegmatis*: Promoter Recognition by Stress-Specific Extracytoplasmic Function Sigma Factors σ^H and $\sigma^{F\nabla}$†

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The survival of a bacterium with a depleted oxygen or nutrient supply is important for its long-term persistence inside the host under stressful conditions. We studied a gene, *dps*, from *Mycobacterium smegmatis*, encoding a protein, Dps (for DNA binding protein from starved cells), which is overexpressed under oxidative and nutritional stresses and provides bimodal protection to the bacterial DNA. Characterization of the *dps* promoter in vivo is therefore important. We cloned a 1-kb putative promoter region of the *dps* gene of *M. smegmatis* in an *Escherichia coli-Mycobacterium* shuttle vector, pSD5B, immediately upstream of the *lacZ* gene. Promoter activities were assayed in vivo both in solid medium and in liquid cultures by quantitative β-galactosidase activity measurements. To characterize the minimal promoter region, a 200-bp fragment from the whole 1-kb sequence was further cloned in the same vector, and in a similar way, β-galactosidase activity was quantitated. Primer extension analysis was performed to determine the +1 transcription start site of the gene. Point mutations were inserted in the putative promoter sequences in the −10 and −20 regions, and the promoter sequence was confirmed. The promoter was not recognized by purified *M. smegmatis* core RNA polymerase reconstituted with purified *Mycobacterium tuberculosis* σ^A or σ^B during multiple- and single-round in vitro transcription assays. Promoter-specific in vivo pull-down assays with an immobilized 1-kb DNA fragment containing the *dps* promoter established that extracellular function sigma factors were associated with this starvation-inducible promoter. Single-round transcription at the *dps* promoter further supported the idea that only core RNA polymerase reconstituted with σ^F or σ^H can generate proper transcripts.

The regulation of gene expression in mycobacteria is an important area of research in order to address the basic biology of a gram-positive organism, as well as to understand the various mechanisms of pathogenicity. Promoter identification and analysis in mycobacteria, therefore, are essential and have improved significantly with the help of various reporter technologies (16). The approach involves in vitro fusion of probable promoter and other regulatory regions just upstream of genes whose products are stable and easily assayable. Such simple systems can be used to monitor the effects of mutations in promoter elements, as well as to follow their responses to various environmental signals. Promoterless plasmids containing the *Escherichia coli lacZ* gene, encoding β-galactosidase (12), have been used as candidates for a reporter assay, with various lactose derivatives as substrates (3, 6, 16, 20, 21).

We attempted here to generate a *lacZ* construct with the *dps* promoter in the *E. coli-Mycobacterium* shuttle vector pSD5B (16). This vector is promoterless to begin with, and thus, when the intrinsic *lacZ* is placed in frame downstream of the *dps* promoter, it can be regulated as a function of the expressibility of the *dps* gene. Dps has been reported to be a nucleoid-like DNA binding protein capable of in vitro oligomerization under optimum conditions (25). It has been identified under glucose-limiting conditions or in stationary phase in mycobacteria (2). Its major function is to protect the genomic DNA under stress encountered during the stationary phase (2, 15). We have shown that DNA protection occurs in two different ways, and we called this bimodal protection (14, 15).

Thus, in our system, it would be interesting to assess what is the minimum promoter element necessary to regulate *lacZ* expression as a function of the glucose concentration in the growth medium. Eventually, it would help to utilize such a promoter element in regulated gene expression in mycobacteria.

We further attempted here to discover the nature of the sigma factor(s) that regulates the transcription of the *dps* gene. Thirteen sigma factors have been identified in mycobacteria (7, 11); however, clear annotation of all the sigma factors in *Mycobacterium smegmatis* is still not available in the The Institute for Genomic Research (TIGR) database, although the sequence of the whole *M. smegmatis* genome is known (33). On the other hand, all 13 sigma factors are identified in the *Mycobacterium tuberculosis* database (TIGR) (31). The sequence homology among various subunits of *M. tuberculosis* and *M. smegmatis* RNA polymerases (Table 1) prompted us to reconstitute a heterologous system of *M. smegmatis* core RNA poly-

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TABLE 1. Comparison of core subunits of RNA polymerase and annotated sigma factors[a]

Subunit name	Species	Global alignment score	CLUSTAL W alignment score	% Identity			
				E. coli		*M. smegmatis*	
				SIM[b]	LALIGN[c]	SIM[b]	LALIGN[c]
α	*M. tuberculosis*	2,035	1,916			90.9	90.9
β		7,943	6,663			91.7	91.4
β′		7,943	7,463			91.4	91.4
ω		539	485			83.0	79.1
σ^A		1,697	2,217	60.3	32.8	79.8	76.7
σ^B		1,475	1,790	43.7	33.7	93.7	92.3
σ^F		972	1,237	29.7	27.8	83.8	80.4
σ^H		202	391	27.5	14.1	38.8	32.6

[a] σ^J, σ^L, σ^M, σ^C, σ^D, σ^K, σ^I, σ^G, and σ^E were found to be putative in the mc^{2}155 genome; hence, sequence homology couldnot be performed.
[b] http://www.expasy.ch/tools/sim-prot.html.
[c] http://www.ch.embnet.org/software/LALIGN_form.html.

merase ($\alpha_2\beta\beta'\omega$) and various *M. tuberculosis* sigma factors to generate a functionally active holo-RNA polymerase that could recognize the *dps* promoter element. It should be mentioned here that the *dps* gene is present in *M. smegmatis* and other mycobacteria, but not in *M. tuberculosis*. We believe that a heterologous transcription system like that shown here can address the basic issues concerning the structure of the promoter element and its regulation and that it is useful. The RNA polymerase used here was genetically modified with a histidine tag at the C-terminal end of the β′ subunit and purified through a single Ni column. The core RNA polymerase was isolated using a phosphocellulose column. The identification of proteins trapped in the immobilized promoter-containing template and subsequent single-round in vitro transcription helped in characterizing the sigma factors responsible for recognizing the *dps* promoter.

MATERIALS AND METHODS

Bacterial strains, medium, and growth conditions. All the plasmids used in the present study are listed (see Table 3). The *M. smegmatis* wild-type strain mc^{2}155 (33) was used in all experiments. The bacteria were grown in MB7H9 medium supplemented with 2% glucose, 25 μg/ml kanamycin, and 0.05% Tween 80. Plate cultures were grown in liquid medium with 1.5% agar. For quantitative β-galactosidase assays in liquid cultures, 2 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was used as a substrate. However, for plate assay of *lacZ*, 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing MB7H9 plates were used.

A transcriptional fusion construct of a putative 1-kb promoter of the *dps* gene and *lacZ* and promoter induction assay. A set of two primers, Prisat1 and Prisat2 (Table 2), was used to amplify the 1-kb fragment containing upstream, as well as downstream, sequence (up to +46 bp) from the +1 start site of mc^{2}155 genomic DNA. The PCR product was digested with XbaI and SphI and was then cloned in a promoterless *E. coli-Mycobacterium* shuttle vector, pSD5B, with a *lacZ* reporter system (16) previously digested with the same set of restriction enzymes. The resulting recombinant plasmid, pSdps1 (Table 3), therefore had the *lacZ* reporter gene just downstream of the *dps* promoter.

This plasmid was then electroporated to mc^{2}155 competent cells, cultured to mid-log phase (optical density at 600 nm, 0.7) in MB7H9 medium supplemented with 2% glucose, harvested, washed once with phosphate-buffered saline, and then transferred to MB7H9 medium supplemented with either 2% or 0.02% glucose, and cells were harvested at different time points as required. Promoter induction was assayed by β-galactosidase activity (*lacZ* expression) in liquid culture using ONPG as described previously (27). The activity was represented in Miller units and was calculated as follows: activity (Miller units) = 1,000 × A_{420}/(time [min] × volume [culture] × optical density at 600 nm).

All the readings were taken in triplicate and averaged. In each case, mc^{2}155 transformed with the empty vector pSD5B was taken as the negative control (data not shown).

Subcloning and characterization of a minimal 200-bp promoter region from the 1-kb promoter. A set of two primers, Prisat200 and Prisat2 (Table 2), was used to amplify the minimal 200-bp promoter fragment, along with the same downstream stretch of sequence from the pSdps1 plasmid. The PCR product was digested with XbaI and SphI and was then cloned in a promoterless *E. coli-Mycobacterium* shuttle vector, pSD5B, with a *lacZ* reporter system (16) previously digested with the same set of restriction enzymes. The resulting recombinant plasmid, pSdps200 (Table 3), therefore had the *lacZ* reporter gene just downstream of the *dps* promoter. Promoter induction under starvation (0.02% glucose) was assayed using liquid cultures of mc^{2}155 cells transformed with pSdps200, similarly to mc^{2}155-pSdps1.

Mapping of the transcription start site by the primer extension method. The +1 transcription start site of the *dps* gene was identified by the primer extension method (15). *M. smegmatis* transformed with pSdps1 was grown for 60 h in MB7H9 medium supplemented with 0.02% glucose and harvested. Total RNA was then isolated using an RNA minikit (Auprep). A total of 10 to 12 μg of RNA was used to prepare cDNA using a primer end labeled with [γ-^{32}P]ATP (Board of Radiation and Isotope Technology, Bombay, India), Map2 (Table 2), and RevertAid Moloney murine leukemia virus reverse transcriptase (Fermentas). Sequencing-grade *Taq* DNA polymerase and dideoxynucleoside triphosphates were obtained from Promega. The primer was designed approximately 71 bp downstream of the +1 site. A sequencing ladder was prepared by the dideoxy-mediated chain termination method and was run using the Fmol DNA cycle-sequencing system (Promega) with an annealing temperature of 50°C. A 10% polyacrylamide gel containing 6 M urea was run to resolve the sequencing product. The gel was dried, and a picture was taken using a phosphorimager (FLA2000; Fujifilm).

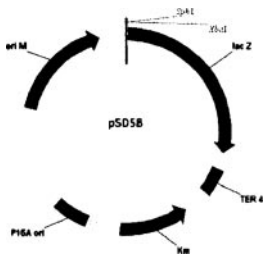
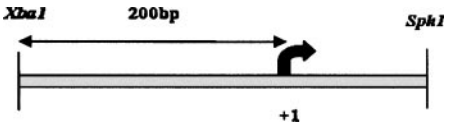
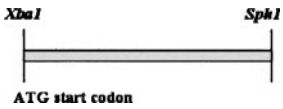
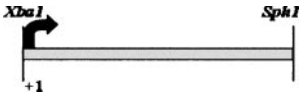
Identification of the promoter region by site-directed mutations of bases in the −10 and −20 regions. Several upstream deletion constructs were made from mc^{2}155 genomic DNA by specific PCR primers in the upstream region proximal to the +1 start site (Table 3). The aim was to discover the minimal promoter sequence for the *dps* gene. The PCR conditions were 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 30 cycles. mc^{2}155 cells transformed with pRCP1, pRCP2, pRCP20, and pRCP31 were grown in liquid MB7H9 medium (0.02% glucose) for 72 h, and promoter activity was assayed similarly by *lacZ* expression. mc^{2}155-pSdps200 was taken as a positive control.

Site-specific point base mutations were further carried out by the Quickchange protocol (Stratagene) in the −20 (TCGAAC) and −10 (GAAACG) regions of the promoter. The PCR conditions were 98°C for 10 s, 65°C for 30 s, and 72°C for 3 min (25 to 30 cycles), using pSdps200 as a template. All mutations were

TABLE 2. Primers used in the present study

Primer name	Sequence (5′–3′)
Prisat1	GGCATCGATCCCCTGCTACCG
Prisat2	GCCACATCGCTCGCCTTCTTG
Prisat200	GGGATGGTCGAGTTGCTCGAAC
dpsrev	CAGCGTCGCGATGCGCTCGGCCACC
Map2	GGAAGTGATTCCTCCGGATATCG

TABLE 3. Plasmid constructs containing several deletion constructs of the *dps* promoter

Plasmid	Length (kb)	Description	Promoter element inserted
pSD5B	9.50	*E. coli-Mycobacterium* shuttle vector containing promoterless *lacZ* gene; Kan^r	
pSdps1	10.50	pSD5B containing 1-kb *dps* upstream region	
pSdps200	9.74	pSD5B containing 200-bp *dps* upstream region	
pRCP1	9.54	Upstream of *dps* gene removed, including the +1 site	
pRCP2	9.55	Upstream of *dps* gene removed, excluding the +1 site	
pRCP20	9.76	Upstream of *dps* gene up to −20 site deleted	
pRCP31	9.77	Upstream of *dps* gene up to −31 site deleted	

confirmed by DNA sequencing. β-Galactosidase activity was assayed in liquid cultures to compare the promoter induction under starvation.

Growth phase-dependent variation of Dps protein in vivo. mc^{2}155 cell pellets were collected at different time points during growth and lysed. Crude cell lysates were resolved on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred electrophoretically to polyvinylidene difluoride membranes (Amersham Pharmacia). The membranes were probed with affinity-purified Dps antibody diluted 2,500-fold. The reactive proteins were identified using a goat anti-rabbit antibody conjugated to horseradish peroxidase. The protein bands were developed with amino ethyl carbazole as the substrate.

Promoter-specific pull-down assay and identification of associated sigma factors. A 1.1-kb linear PCR-amplified *dps* promoter was incubated at 37°C for 45 min with 10 to 20 μM biotin-11-dUTP (Fermentas), 100 μM deoxynucleoside triphosphate mix (Sigma), and 0.6 unit of Klenow fragment (Fermentas) in buffer A (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.2 mM EDTA), and unincorporated biotin-dUTP and deoxynucleoside triphosphates were removed by ethanol precipitation. Biotinylated *dps* promoter fragments were pooled in 25 μl of double-distilled water. Twenty-five microliters of biotin-*dps* DNA fragments was then mixed with 250 μl of streptavidin-agarose beads (Sigma) for 1 hour at room temperature with constant stirring. Biotinylation of DNA was checked by characteristic UV and fluorescence measurements. The ratio between biotin and DNA was found to be nearly 1:1. Crude cell lysates were taken from wild-type mc^{2}155 cells and grown for 72 h in 0.02% glucose-supplemented MB7H9 medium, and then DNA binding proteins were precipitated with polyethyleneimine. Protein extraction steps from the pellet were performed by 0.4 M ammonium sulfate precipitation, and the pellet obtained in this step was dissolved in buffer B (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM potassium glutamate, 10% glycerol, 0.5 mM EDTA, and 0.1% Triton X-100) with 10 μl of *dps*-biotin-streptavidin-agarose beads and kept for binding overnight at 4°C with constant mixing. The beads were allowed to settle on ice for 10 min, the supernatant was removed, and the beads were washed three times with buffer B. Bound proteins were then eluted with buffer C (20 mM Tris-HCl, pH 7.9 at 4°C, 100 mM NaCl, 0.2 mM EDTA, and 0.1 mM dithiothreitol [DTT] [TGED] plus 0.4 M NaCl) and were subjected to Western blotting with sigma antibodies (obtained from Astrazeneca) and mass spectrometric identification.

Reconstitution of RNA polymerase holoenzymes from *M. tuberculosis* SigF and SigH with core RNA polymerase from *M. smegmatis*. In vitro purification of His_6-tagged holo-RNA polymerase (obtained from strain SM07, made by Raju Mukherjee) from *M. smegmatis* (24 h in 2% glucose plus MB7H9 medium) and N-terminal His_6-tagged SigH from *M. tuberculosis* (in pRSET-a vector [Astrazeneca]) was performed using the Qiagen Ni-nitrilotriacetic acid affinity matrix according to the manufacturer's instructions. For this purpose, RNA polymerase was first purified from 500 ml of actively growing culture of *M. smegmatis*, following the same protocol as reported earlier (26) up to polyethyleneimine precipitation, and then by ammonium sulfate fractionation. Subsequently, the partially purified enzyme fractions were passed through a Qiagen Ni-nitrilotriacetic acid column for affinity purification (details of this preparation will be published elsewhere). N-terminal glutathione *S*-transferase-tagged SigF from *M. tuberculosis* (in the pGEX-3X vector [Astrazeneca]) was also purified using a glutathione-Sepharose affinity matrix. All of the proteins were dialyzed in TGED

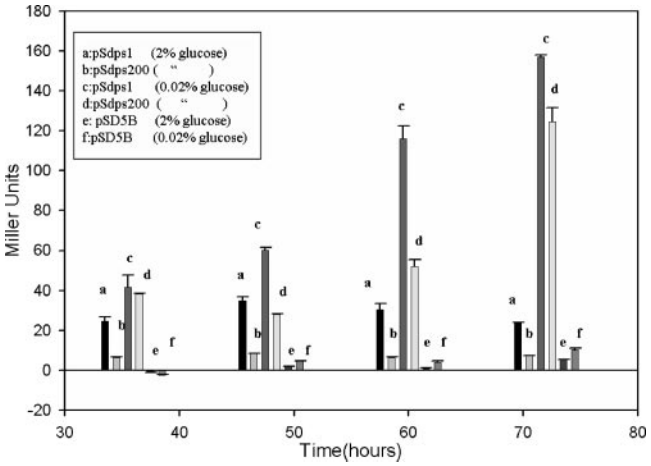


FIG. 1. Comparison of promoter induction of pSdps1 (bars a) and pSdps200 (bars b) in MB7H9 medium supplemented with 2% glucose and 0.02% glucose, pSdps1 (bars c), and pSdps200 (bars d). The error bars indicate standard deviations.

plus 50% glycerol. Purification of core RNA polymerase from the His_6-tagged holoenzyme was carried out on a phosphocellulose ion-exchange column (P11; Whatman) equilibrated with TGED and 50 mM KCl. The flowthrough and wash fractions contained σ^A, and the core subunits were bound to the matrix. Elutions were done in TGED plus 500 mM KCl when the core polymerase ($\alpha_2\beta\beta'\omega$) was eluted out. The eluted fractions were checked in 10% SDS-polyacrylamide gel electrophoresis (PAGE) and by Western analysis with σ^A antibody. A reconstitution experiment was performed by incubating the *M. smegmatis* core RNA polymerase and *M. tuberculosis* σ^A, σ^F, and σ^H at 1:2 molar ratio in buffer R (50 mM Tris-HCl, pH 7.9 at 4°C, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA) at 37°C for 30 min and were resolved in an 8% native PAGE and silver stained. The reconstituted holoenzyme bands were then cut out and subjected to SDS-PAGE analysis and also silver stained.

In vitro transcription assay. A multiple-round in vitro transcription was performed to measure the RNA polymerase activity as described previously (23). The assay mixture contained 40 mM Tris-HCl, pH 8.0; 200 mM NaCl; 10 mM $MgCl_2$; 0.1 mM EDTA; 14 mM 2-mercaptoethanol; 200 μM (each) of ATP, CTP, and GTP; 50 μM UTP; 2 μCi of [^{3}H]UTP (Perkin Elmer); and 1.5 μg calf thymus DNA per 100 μl. The calf thymus DNA assay mixture was used as a control for nonspecific transcription. An assay mixture containing σ^A-specific acetamidase gene promoter DNA (2 kb) was used as a positive control for specific transcription. DNA (1 kb) containing the *dps* promoter was then subjected to specific transcription with RNA polymerase of *M. smegmatis* containing sigma A, F, and H from *M. tuberculosis* (at a DNA/holopolymerase molar ratio of 1:10). The enzymes were all incubated with the assay mixture for the optimum time at 37°C and then spotted on DE-81 papers (Whatman) presoaked in 5 mM EDTA to stop the reaction. The dried filters were washed twice in 5% Na_2HPO_4 buffer for 15 min, twice in deionized water for 5 min, and finally once with ethanol and dried. The filters were then placed into scintillation vials filled with Ultima Gold scintillation fluid (Packard) and counted.

An in vitro single-round runoff transcription assay was performed using conditions modified from those described earlier (18). Purified σ^F, σ^H, and σ^A proteins from *M. tuberculosis* were incubated with *M. smegmatis* core RNA polymerase in a 2:1 molar ratio at 37°C for 30 min in transcription buffer [500 mM Tris-HCl, pH 7.8 at 37°C, 30 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT, 250 μg/ml bovine serum albumin (nuclease free), 500 mM NaCl]. To the reconstituted holoenzymes, DNA template (at a protein/DNA molar ratio of 10:1) and a mixture of heparin, [α-^{32}P]UTP, and four NTPs were added, and the mixture was incubated for 10 min at 37°C. The concentrations of nucleotides and heparin in the final reaction volume were 0.15 mM ATP, 0.15 mM GTP, 0.15 mM CTP, 0.15 mM UTP, 2 μCi [α-^{32}P]UTP, and 200 μg heparin/ml. The DNA template for in vitro transcription was prepared by PCR using primers Prisat1 and dpsrev (Table 2), with mc^2155 genomic DNA as a template. A linear fragment of 317 bp, which generated a transcript of 234 bp, was subsequently used for the assay. Known σ^F- and σ^H-dependent promoters, *usfXp*$_1$ (in pYZ99 vector; Bose Institute, Kolkata, India) and *sigBP* (in pARC8176 vector [Astrazeneca]), respectively, were used as positive controls for in vitro transcription. All samples were electrophoresed in a 10% denaturing polyacrylamide gel containing 6 M urea and analyzed by phosphorimager (FLA2000; Fujifilm).

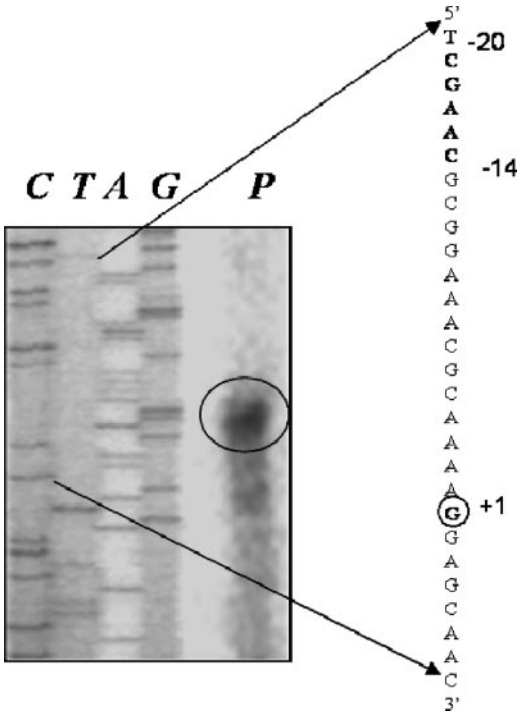


FIG. 2. Identification of the +1 transcription start site by primer extension analysis. Total RNA was isolated from *M. smegmatis* cells transformed with pSdps1 and grown for 72 h in 0.02% glucose-supplemented MB7H9 medium. The cDNA was synthesized and run in lane P, along with a sequencing reaction of the top strand with a reverse primer represented by C, T, A, and G. The sequence is shown on the right, and the +1 site is circled.

RESULTS

The full-length 1-kb *dps* promoter and minimal 200-bp *dps* promoter fragment show comparable promoter activities under glucose starvation. The 1-kb upstream region of the *dps* gene, when fused to the upstream region of the *lacZ* reporter in a promoterless *E. coli-Mycobacterium* shuttle vector, pSD5B (16), showed significant promoter activity in mc^2155 cells. The promoter activity was observed only under glucose starvation, i.e., in 0.02% glucose-supplemented MB7H9 medium, and was maximal around 60 to 72 h of growth (Fig. 1, bars c). It was fivefold more than that in 2% glucose (Fig. 1, bars a). The quantitation of the promoter activity was carried out in liquid cultures through ONPG activity assays. It was then calculated in terms of Miller units (see Materials and Methods) (27). All the experiments were performed under identical conditions in triplicate. Next, a promoter-reporter construct, pSdps200 (Table 3), was made by PCR amplification of the 200-bp minimal upstream promoter fragment using a set of two primers, Prisat200 and Prisat2 (Table 2), in pSD5B. Promoter activity assays were followed in liquid cultures similarly to that of mc^2155-pSdps1. From Fig. 1, it is evident that this region was sufficient to show significant promoter induction, comparable to that observed with the 1-kb promoter region, under starvation conditions (Fig. 1, bars d). No significant promoter induc-

A

Name	-10	-20
pSdps200	GAAACG	TCGAAC
pSD101	**T**AAACG	
pSD103	GA**C**ACG	
pSD106	GAAAC**T**	
pSD201		**G**CGAAC
pSD203		TC**T**AAC
pSD206		TCGAA**T**

B

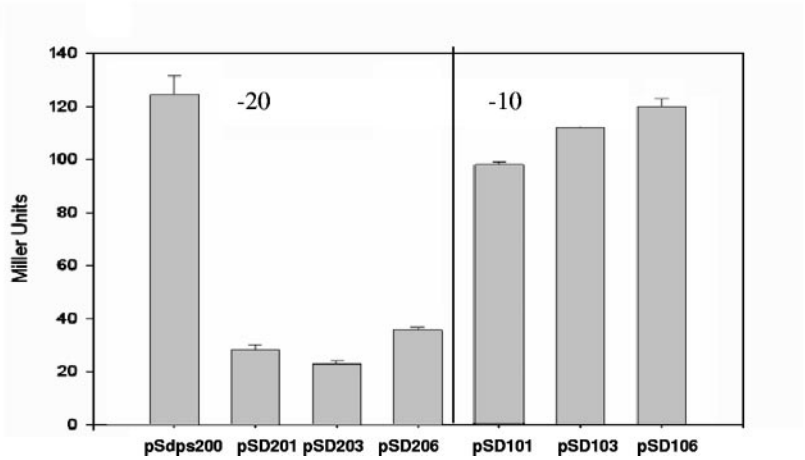


FIG. 3. (A) Promoter sequence as identified by site-directed mutagenesis of pSdps200; point base mutations in the −10 and −20 regions are shown in boldface and underlined. (B) Effects of specific mutations (first, third, and sixth bases) in −10 and −20 upstream sequences from the +1 site of the *dps* gene on promoter activity. Differential *lacZ* expression in liquid cultures of *M. smegmatis* transformed with variants of pSdps200 is shown. The error bars indicate standard deviations. The cells were grown in MB7H9 medium supplemented with 0.02% glucose and harvested at 72 h.

tion was found in 2% glucose-supplemented MB7H9 medium (Fig. 1, bars b).

Identification of the transcription start site (+1) by primer extension analysis. Primer extension analysis was performed to map the +1 transcription start site of the *M. smegmatis dps* gene (Fig. 2). Here, the transcription initiation site (+1) was found to be a "G" base that was 8 bases upstream from the *dps* translation start codon, ATG. The sequence had been read in a reverse complementary way, as a reverse primer specific to the vector backbone was used, and the cDNA (Fig. 2, lane P) was around 93 nucleotides in length.

Characterization of the promoter sequence. In order to identify the upstream promoter region of the *dps* gene, we made several upstream deletion constructs (Table 3). pRCP20 and pRCP31 were generated by removing all the bases upstream up from the putative −20 bases and −31 bases, respectively. This was achieved by keeping the position the same for the reverse primer at the 3′ end while shifting the forward primer to different locations. In all cases, mc^2155 genomic DNA was used as the template during PCR. Two negative controls were also constructed, one of which contained no bases upstream from the +1 start site (pRCP1) and hence was equivalent to the empty vector, pSD5B, while the other one had only the +1 transcription site (pRCP2) (Table 3). An ONPG activity assay was performed in 0.02% glucose-supplemented MB7H9 medium. The results (see Table S1 in the supplemental material) indicated significant promoter induction with pRCP20. The promoter containing upstream −20 base pairs was therefore sufficient to give activity comparable to that of pSdps200 and pRCP31. Cells transformed with pSD5B, pRCP1, and pRCP2 did not show any activity, as expected. Point base mutations in these upstream regions were then carried out at every first, third, and sixth base of the −20 (TCGAAC) and −10 (GAAACG) sequences (Fig. 3A) by site-directed mutagenesis (see Materials and Methods). All of the single-base mutation constructs are listed in Fig. 3A. As is evident from Fig. 3B, mutations in the hexameric stretch TC GAAC (−20) severely affected *lacZ* expression, as observed in ONPG assays in liquid cultures of the bacteria grown for 72 h in 0.02% glucose (lanes b, c, and d). This indicated that the *dps*

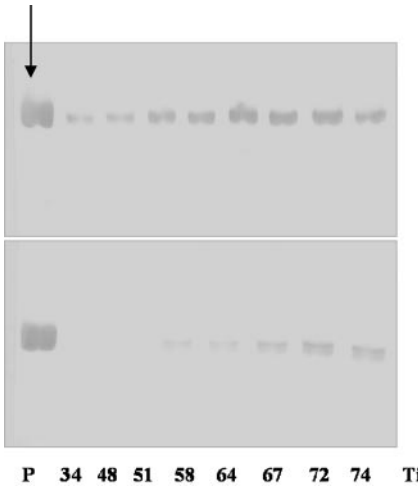


FIG. 4. Growth phase-dependent intracellular expression profile of Dps protein in wild-type mc^{2}155 cultures under glucose-starved (0.02%) and -fed (2%) conditions in MB7H9 medium. P, in the first lane (indicated by the arrow), represents pure His_6-tagged Dps protein as a positive control.

gene is controlled by a promoter element located 20 bases upstream from the +1 start site.

In a separate set of experiments, we looked at the Dps protein expression profile at different time points of bacterial growth under glucose-fed, as well as glucose-starved, conditions. Figure 4 shows the protein expression levels at different stages of growth probed with anti-Dps antibody. It was clear that Dps, being a starvation-induced protein, was expressed in late stationary phase, which more or less mimicked starvation conditions, as most of the carbon source was used up; however, the expression was basal under carbon-fed conditions. Therefore, we concluded that Dps was induced under glucose starvation at both the transcriptional and translational levels.

The *dps* promoter is not recognized by either σ^A or σ^B: a new set of ECF sigma factors is required. We observed initially that there was no transcription signal during in vitro reaction at the *dps* promoter with purified *M. smegmatis* holo-RNA polymerase containing the principal sigma factor (1, 4). This raised the question of whether any other sigma factors are necessary to recognize the *dps* promoter. However all extracellular function (ECF) sigma factor genes are not yet annotated for the *M. smegmatis* genome in the TIGR database. Thus, we first developed a pull-down assay over an immobilized DNA template in order to identify the nature of the sigma factors associated with the transcription complex. Subsequently, in vitro transcription at the *dps* promoter was carried out with *M. smegmatis* core RNA polymerase reconstituted with *M. tuberculosis* sigma factors.

We first attempted to immobilize the DNA template by incorporating a biotinylated nucleotide at its terminal. The biotin-avidin interaction is known to be one of the strongest noncovalent interactions, with a K_a (association constant) value of 10^{13} to 10^{15} M^{-1} (13), and has wide application in protein-DNA interactions. Biotinylation of a PCR-amplified 1-kb promoter fragment was carried out with Klenow polymerase, and the labeled promoter was further immobilized over streptavidin-agarose beads. Subsequently, mc^{2}155 cells were

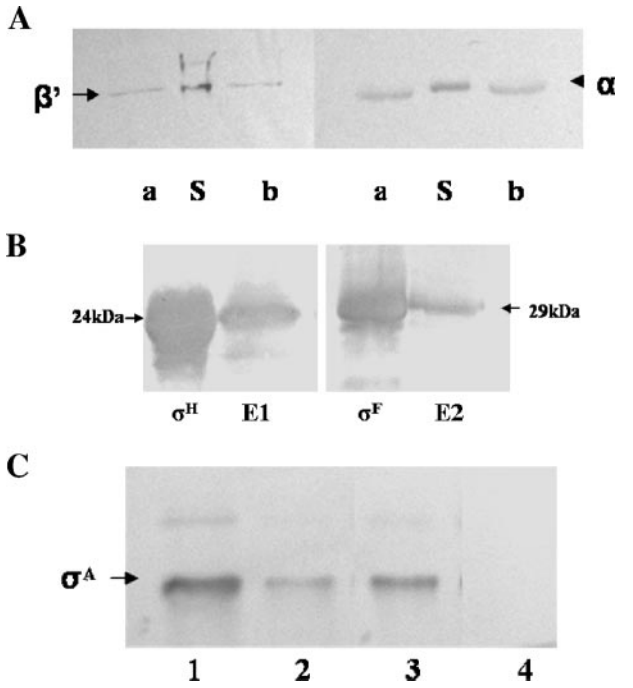


FIG. 5. (A) Identification of the core RNA polymerase subunits in the eluted fractions of biotin-streptavidin pull-down assays with the *dps* promoter at 48 (lanes a) and 72 (lanes b) hours under glucose-depleted conditions. The eluates were probed with *M. tuberculosis* β′ antibody and *M. smegmatis* α antibodies. In both cases, lanes S contain the purified holo-RNA polymerase as a standard. (B) Identification of new ECF family sigma factors σ^H and σ^F associated with the *dps* promoter by promoter-specific biotin-streptavidin pull-down assays. The eluted fractions, E1 and E2, were treated with antibodies against *M. tuberculosis* σ^H and σ^F, respectively. (C) Western blot with σ^A antibody done to check the sensitivity of the *dps* promoter with holo-RNA polymerase containing σ^A. Lane 1, pure σ^A; lanes 2 to 4, pull-down fractions with the 1.6-kb *rel* promoter (lane *2*), the 2-kb acetamidase gene promoter (lane 3), and the 1-kb *dps* promoter (lane 4).

grown in MB7H9 medium supplemented with 0.02% glucose and harvested at 72 h. DNA binding proteins were precipitated with 10% polyethyleneimine and further fractionated by ammonium sulfate precipitation. The total protein mixture was then incubated with the *dps*-biotin-streptavidin beads, associated proteins were eluted out, and the fractions were subjected to immunoblotting against *M. smegmatis* α and *M. tuberculosis* β′ and sigma antibodies. As is evident from Fig. 5A, the core subunits were present and two ECF sigma factors, σ^F and σ^H, were detectable in the sample (Fig. 5B, E1 and E2). These sigma factors are known to be involved in persistence and heat shock and oxidative stress responses in *M. tuberculosis* (5, 24, 30). We also noticed growth phase-dependent expression of σ^F and σ^H in *M. smegmatis* culture under 0.02% glucose conditions, but not when the culture was grown in 2% glucose (not shown), further supporting our previous results. To further confirm that *dps* was not recognized by σ^A, pull-down assays were performed with two other promoters, those for *rel* (17) and the acetamidase gene (28), and probed with σ^A antibody. It is clear from Fig. 5C that σ^A was not present with the *dps* promoter, whereas both *rel* and the acetamidase gene were σ^A specific, which further supported our contention that *dps* was

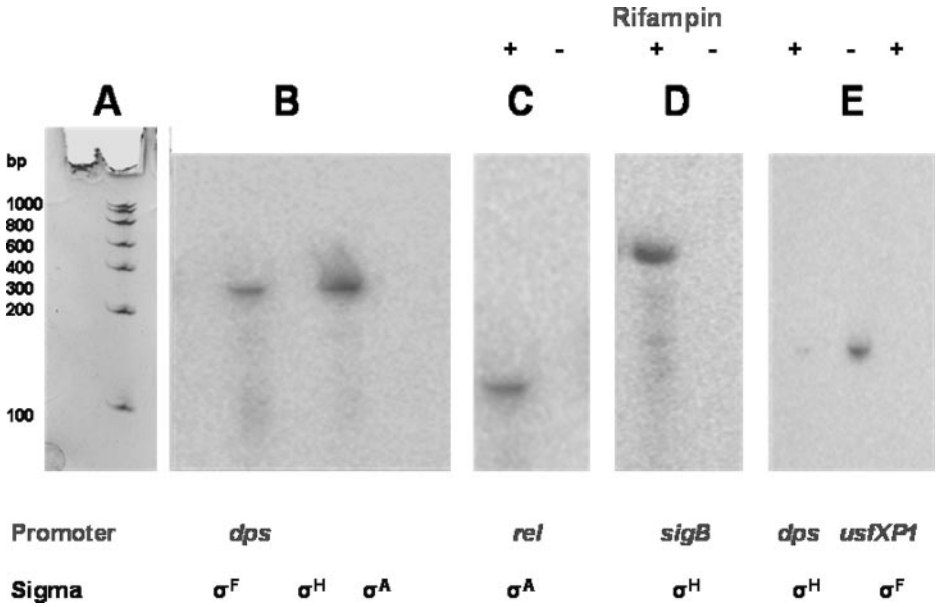


FIG. 6. Single-round heparin-resistant runoff transcription at the *dps* promoter carried out with *M. smegmatis* holo-RNA polymerase. (A) A 10% polyacrylamide gel containing 6 M urea shows an RNA ladder run separately and matched. (B) *M. tuberculosis* σ^A, σ^F, and σ^H were reconstituted with *M. smegmatis* core RNA polymerase to obtain the reconstituted holopolymerases. The intensity of each transcript band as obtained from phosphorimager analysis showed the expected transcripts for σ^F (right lane) and σ^H (middle lane). No transcripts were obtained with σ^A-reconstituted RNA polymerase (left lane). (C) In order to test single-round runoff transcription with σ^A-reconstituted RNA polymerase, the *rel* promoter was used as a positive control. The left lane shows the presence of the transcript, and the right lane shows its absence with rifampin (0.1 μg/ml). (D) Positive controls are shown using the known *M. tuberculosis* σ^H-dependent *sigB* promoter in the absence (left lane) and in the presence (right lane) of rifampin, as well as the σ^F-dependent $usfXp_1$ promoter in the absence (middle lane) and in the presence (right lane) of rifampin. No band was obtained from the *M. smegmatis dps* promoter in the presence of rifampin with σ^H-reconstituted RNA polymerase (left lane).

recognized directly by stationary-phase-induced ECF sigma factors. It should be mentioned here that we probed the pull-down assay eluate in the same way with other sigma factor antibodies from *M. tuberculosis*, and none of them showed any positive result.

Multiple- and single-round in vitro runoff transcription assay with σ^F-, σ^H-, and σ^A-reconstituted RNA polymerases. To determine whether the *dps* promoter is recognized by σ^F- or σ^H-containing RNA polymerases, the following multiple- and single-round transcription assays were carried out. Reconstitution of *M. smegmatis* core RNA polymerase with the *M. tuberculosis* sigma factors A, F, and H resulted in a protein band upon 8% native PAGE analysis. The band, when subjected to 12% SDS-PAGE analysis, showed the presence of all the core subunits, along with the sigma factors with which they were reconstituted (see Fig. S1 in the supplemental material). We carried out multiple-round transcription assays at the *dps* promoter with reconstituted RNA polymerases, as described in Materials and Methods. The amounts of [^{3}H]UTP incorporated in the transcripts were monitored to quantify the transcription activities. No significant transcription was observed at the *dps* promoter when it was incubated with σ^A-reconstituted polymerase, whereas the acetamidase gene resulted in appreciable radioactive signal. Transcription from the *dps* promoter, on the other hand, was initiated by both σ^F- and σ^H-containing polymerases (see Fig. S2 in the supplemental material).

When the *dps* promoter containing linear DNA was subjected to single-round runoff transcription in the presence of heparin (18), an expected length of 234 bp was noticed with σ^F- and σ^H-containing core RNA polymerase (Fig. 6B). It is also clear from the figure that σ^A-containing RNA polymerase is incapable of initiating transcription at this promoter. In addition, in vitro transcriptions with known *M. tuberculosis* σ^A-, σ^F-, and σ^H-dependent promoters, *rel* (17), $usfXp_1$ (9), and *sigB* (30), respectively, were performed as positive controls, and as is evident from Fig. 6C, D, and E, they indeed showed runoff transcripts of the expected lengths. Also, in the presence of rifampin, the transcription reactions were all found to be inhibited (Fig. 6), thereby confirming the formation of true RNA transcripts in all cases. These experiments conclusively established that the *dps* promoter can be transcribed only by ECF sigma factors, σ^F and σ^H.

DISCUSSION

The principal aim of this study was to characterize a starvation-induced mycobacterial transcription system in order to understand the mechanism regulating gene expression when bacteria enter stationary phase and eventually encounter different stresses, like oxygen and nutrient depletion and heat shock. The stationary-phase-induced expression of the *dps* gene and its regulation were studied extensively in *E. coli* (22). Various other stresses had also been shown to induce *dps* expression at the transcriptional or translational level (2, 25, 34). In *E. coli*, *dps* expression was found to be regulated by OxyR under oxidative stress, whereas σ^S is required for induction under starvation in stationary phase (2, 25). Here,

FIG. 7. Comparison of promoter consensus sequences identified by σ^F and σ^H factors in the known $usfXp_1$ and *sigB* promoters from *M. tuberculosis*, respectively, and the *dps* promoter from *M. smegmatis* (this study).

we have shown that the *dps* promoter of *M. smegmatis* is induced under glucose starvation conditions and that the level of induction increases as bacteria enter the stationary phase. Under glucose-fed conditions, however, induction was negligible.

In *E. coli*, it is known that the ClpXP protease system degrades the mature Dps protein, thereby regulating the intracellular level of Dps at the posttranscriptional level. In mycobacteria, however, such negative-feedback control systems are not known. In all our reporter assays, we electroporated the promoter plasmid constructs into mc^2155 cells, which already have a copy of the full-length *dps* gene in the bacterial DNA. The protein is therefore expressed from early exponential phase under starvation conditions, as well as after 60 h under glucose-fed conditions, to exert any positive control. In 2% glucose, however, significant promoter induction was not seen in any of the constructs (Table 3 and Fig. 1), so perhaps there was no positive-feedback control associated with promoter induction at the posttranscriptional level.

Systematic study of the mapping of the promoter element resulted in a putative hexameric consensus sequence, TCG AAC. The *dps* promoter-specific biotin-streptavidin pull-down assay (10, 19, 35) presented here identified a set of two stress-responsive sigma factors, F and H (Fig. 5B), associated with it. Later, growth-dependent expression of these two sigma factors was also monitored in mc^2155 cell lysates, where concomitant expression of both proteins under stationary-phase conditions, along with Dps protein, was observed. However, sigma H expression was more than that of σ^F. This was indeed interesting, as it further supported the idea that bacteria adapt to various stresses encountered under dormant conditions by regulation of different subsets of genes and that ECF sigma factors play a crucial role at this stage.

Here, we have shown the purification and reconstitution of holo-RNA polymerase from *M. smegmatis* core enzyme and sigma factors from *M. tuberculosis* (32). This was necessary, as different ECF sigma genes in *M. smegmatis* are still not annotated. However, sequence homology among various sigma factors from mycobacteria and specific transcripts obtained using the heterologous enzymes indicated that such experiments are dependable, at least in vitro.

Recently, it has also been reported that the *M. tuberculosis* *sigB* promoter can be identified by both σ^F and σ^H; however, transcription initiates from different +1 sites (8). Reconstituted enzymes showed the specific single-round runoff transcripts at the *dps* promoter with σ^F and σ^H, whereas other controls showed σ^A-dependent transcription. We tried a sequence-based comparison of the consensus promoter sequences for σ^F and σ^H available in the literature (8, 9, 24, 29) with that obtained for the *dps* promoter in this study (Fig. 7), and more similarities were found with σ^H-dependent promoters (30). Our observation is biologically significant for two main reasons. First, we have developed an efficient single-round in vitro transcription with His-tagged reconstituted RNA polymerase, which can be regulated as a function of glucose or salt concentration in the medium. We believe that this will help a great deal in studying mycobacterial transcription, both in vivo and in vitro, and even at the single-molecule level. On the other hand, reconstitution of heterologous transcription machinery with the *M. smegmatis* promoter and core polymerase and *M. tuberculosis* sigma factors indicates the identical natures of RNA polymerase subunits, which could eventually help in understanding the structure-function relationships of this important enzyme.

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