The gene encoding P27 lipoprotein and a putative antibiotic-resistance gene form an operon in *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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P27 is an antigenic membrane lipoprotein synthesized by members of the *Mycobacterium tuberculosis* complex. Northern blotting and RT-PCR experiments indicated that the genes encoding P27 and a putative antibiotic transporter (P55) form an operon. A promoter region was identified and characterized by deletion analysis in *Mycobacterium smegmatis*. Two transcription initiation points were mapped in *Mycobacterium bovis* BCG by primer extension analysis to 76 bp and 87 bp upstream of the ATG initiation codon. Putative –10 and –35 promoter consensus sequences associated with these showed 66% similarity to previously identified mycobacterial promoters. These results suggest that the P27/P55 operon is transcribed from two promoters in *M. bovis* BCG.

Keywords: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, promoter, operon, lipoprotein

INTRODUCTION

The identification and characterization of mycobacterial antigenic proteins are essential for the development of new diagnostic tests and vaccines, and for the understanding of the pathogenic mechanisms of mycobacteria and the immune responses to them. Numerous mycobacterial antigens have been described (Young *et al.*, 1992); many of them have been expressed in *Escherichia coli* and their recognition by immune cells and antibodies determined. However, little is known concerning their cellular function in mycobacteria. Gene expression regulation studies may help to understand their physiological function, but there is slow progress in this field. Only a few mycobacterial promoters have been studied (Bashyam *et al.*, 1996; Mulder *et al.*, 1997), and many of these are from phage or antibiotic-resistance genes, which might not be similar to the genes involved in virulence. In addition, most mycobacterial promoters described seem to be different from the *E. coli* consensus –10 and –35 elements (Dale & Patki, 1990). Also, little is known about to what extent mycobacterial genes form parts of operons. Some operons have been experimentally demonstrated (Papavinassasundaram *et al.*, 1997; Berthet *et al.*, 1998), but most were postulated

based on nucleotide sequence examination (Braibant *et al.*, 1996a, b; DeMaio *et al.*, 1997; Cole *et al.*, 1998).

P27 is a novel *Mycobacterium bovis* antigen, identified from an expression library using sera from naturally infected cattle (Bigi *et al.*, 1997). Sequence analysis indicated that P27 has a characteristic signal sequence for lipoprotein modification (a signal peptidase type II site). Cellular fractionation experiments suggested that P27 is an integral membrane protein. Downstream of the P27 gene there is an ORF for a 55 kDa protein (P55) which is highly homologous to membrane proteins related to antibiotic resistance in *Streptomyces* and other bacteria. These genes seem to be organized as an operon, because only six bases separate the end of the P27 gene from the start of the P55 gene. The present study demonstrates the polycistronic nature of both genes, as well as the location of the promoter.

METHODS

Bacterial strains, and growth and electroporation conditions.

All cloning steps were performed in *E. coli* DH5 α . Expression studies were done in *Mycobacterium smegmatis* mc²155. Cultures of *E. coli* were grown in LB broth or on LB agar. For the promoter deletion experiments, *M. smegmatis* cells were

Table 1. Plasmids used in the present study

Plasmid	Characteristics*	Reference
pBluescript II SK	Ap ^r , IPTG-inducible <i>E. coli</i> expression vector	Stratagene
pMBA21	Ap ^r , 2.0 kb <i>EaeI</i> fragment of <i>M. bovis</i> AN5 DNA in pBluescript II SK, containing P27 and part of P55	Bigi <i>et al.</i> (1997)
pGEM-5Zf(+)	Ap ^r , <i>E. coli</i> cloning vector	Promega
pGEM-T	Ap ^r , PCR products <i>E. coli</i> cloning vector	Promega
pP27G	Ap ^r , 1.5 kb <i>SacI</i> fragment from pMBA21 cloned into pGEM-5Zf(+)	Present study
pSUM39/41	Km ^r , shuttle cloning vectors	Ainsa <i>et al.</i> (1996)
pMBA27	Km ^r , 1.2 kb <i>PstI</i> fragment from pP27G inserted in pSUM39	Present study
pGdir27	Ap ^r , 2.1Dir/2-1Av3 PCR fragment cloned into pGEM-T	Present study
pG292	Ap ^r , U292/2-1Av3 PCR fragment cloned into pGEM-T	Present study
pG241	Ap ^r , U241/2-1Av3 PCR fragment cloned into pGEM-T	Present study
pMBA27ΔP	Km ^r , 1.011 kb <i>PstI</i> fragment from pGdir27 inserted in pSUM41	Present study
pMBA2792	Km ^r , 1.093 kb <i>PstI</i> fragment from pG292 inserted in pSUM41	Present study
pMBA2741	Km ^r , 1.144 kb <i>PstI</i> fragment from pG241 inserted in pSUM41	Present study
pHP45	Ap ^r /Sm ^r , cloning vector	Prentki & Krisch (1984)
pMBA2792Ω	Km ^r /Sm ^r , 2 kb <i>HindIII</i> fragment from pHP45 inserted in pMBA2792	Present study
pMV261	Km ^r , mycobacterial expression vector	Stover <i>et al.</i> (1991)
pMBA28	Km ^r , 1.011 kb <i>PstI</i> fragment from pMBA27ΔP inserted in pMV261	Present study

* Ap^r, resistance to ampicillin; Km^r, resistance to kanamycin; Sm^r, resistance to streptomycin.

grown to late-exponential phase at 37 °C, either in liquid or on solid LB medium and in Middlebrook 7H11 medium supplemented with OADC (albumin, dextrose, catalase and oleic acid; Difco). When necessary, appropriate antibiotics were added at the following concentrations: ampicillin, 100 µg ml⁻¹; streptomycin, 10 µg ml⁻¹; kanamycin, 50 µg ml⁻¹ for *E. coli* or 20 µg ml⁻¹ for *M. smegmatis*. *M. bovis* BCG Pasteur P1732 was grown to saturation in Middlebrook 7H9/0.05% Tween 80 supplemented with OADC (M7H9-OADC-Tw). Then, 2 ml culture was transferred to 100 ml M7H9-OADC-Tw broth and cultured further for 10 d. Mycobacterial strains were electroporated using the method of Wards & Collins (1996). Electroporations were performed using a Bio-Rad Gene Pulser. For use in transformation, *M. smegmatis* mc²155 was grown to mid-exponential phase in M7H9-OADC-Tw. Following electroporation, *M. smegmatis* was cultured on Middlebrook 7H11 medium supplemented with OADC and containing antibiotic.

DNA manipulations. Standard methods were used for restriction-endonuclease digestion of plasmids, DNA ligations, Southern blotting, and other manipulations (Ausubel *et al.*, 1996). Plasmid DNA isolation was performed using a Wizard Minipreps SV kit, according to the manufacturer's instructions (Promega). DNA probes for Southern blot hybridization were labelled with [α -³²P]CTP using the

Oligolabelling random priming kit according to the manufacturer's instructions (Pharmacia).

Mycobacterial DNA preparation. DNA from *M. bovis* and *M. smegmatis* was prepared according to van Soolingen *et al.* (1991).

Plasmid constructions. Plasmids used are described in Table 1. A 1.5 kb *SacI* fragment was obtained by digestion of pMBA21 and cloned into *SacI*-linearized pGEM-5Zf (Promega), generating pP27G. A 1.2 kb *PstI* fragment of pP27G, corresponding to the 5' non-coding region followed by the sequence encoding P27, was cloned into the *PstI*-digested pSUM39 shuttle vector. The resulting plasmid was named pMBA27.

To obtain plasmids carrying different deletions in the 5' non-coding sequences of the P27 gene, four DNA fragments containing the P27 gene plus different DNA extensions upstream of the ATG start codon were obtained by PCR, using chromosomal DNA from *M. bovis* BCG as template and the 2-1Av3 primer in combination with the 2-1Dir, U292 and U241 primers (Table 2). The PCR products were cloned into the pGEM-T plasmid (Promega), generating pGdir27, pG292 and pG241, respectively. Subsequently, the *PstI* fragments obtained by digestion of pGdir27, pG292 and pG241 were cloned in the *PstI* site of pSUM41 vector, generating pMBA27ΔP, pMBA2792 and pMBA2741, respectively. In parallel, pHP45 was digested with *HindIII* to isolate the

Table 2. Oligonucleotide primers

Primer	DNA sequence	Relative position to P27 start codon (nt)
2-1Dir	5'-cctcacagacaccctctacg-3'	-28
2-1Av3	5'-tgcgctgcaccacaaatc-3'	1116
2-1Av4	5'-gtgacgatccaggtgatcc-3'	870
U292	5'-cgttcctcaacaattccg-3'	-110
U241	5'-atggtcagccgcgcgca-3'	-160
L358P27	5'-tgagttggcggcgccgag-3'	-25
2-1Rev	5'-tcaaccagcgtcaccatcag-3'	1589

streptomycin-resistance gene (2.0 kb), which was subsequently ligated into *Hind*III-linearized pMBA2792. This plasmid was called pMBA2792 Ω . The *Pst*I fragment from pMBA27 Δ P was subcloned into the unique *Pst*I site of pMV261, downstream of the *hsp60* promoter. The resulting plasmid was named pMBA28.

PCR amplification and sequencing. Primers used are listed in Table 2.

PCR amplification was performed with *Taq* DNA polymerase (Promega) under standard conditions in a volume of 50 μ l. The concentration of dNTPs was 0.2 mM each, and 20 pmol of each primer was used. Cycling conditions were: one cycle of 95 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 49 °C (for 2-1Av3/U292 primer set), 53 °C (for 2-1Av3/U241 primer set) or 54 °C (for 2-1Av3/2-1Dir primer set) for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. A total of 0.1 ng plasmid or 2 ng genomic *M. bovis* DNA was used as template. Sequencing was performed by PCR-mediated *Taq*-cycle sequencing using the *fmol* sequencing kit (Promega) according to the manufacturer's instructions. Sequencing products were developed on 6% polyacrylamide gels.

RNA preparation. Total RNA from *M. bovis* BCG and *M. smegmatis* was isolated following the method of Bashyam *et al.* (1996). The two rRNA species and a band of lower molecular mass, corresponding to tRNA, were visible after staining agarose gels with ethidium bromide, indicating that the RNA preparations were of high integrity.

Northern blotting. RNA samples were run on 0.8% agarose gels containing 2.2 M formaldehyde (Ausubel *et al.*, 1996), and transferred by vacuum in 10 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate) to a nylon membrane. RNA was fixed to the membrane by UV light cross-linking. Membranes were pre-hybridized for 3 h in 500 mM sodium phosphate (pH 7.2)/7% SDS at 55 °C. Hybridization was performed as described by Harth *et al.* (1996) in the same buffer at 55 °C for 48 h with an antisense oligonucleotide (L358P27) labelled with [γ -³²P]ATP at 10⁸ c.p.m. μ g⁻¹. Membranes were then washed three to five times with 50 mM sodium phosphate (pH 7.2)/0.1% SDS, and exposed to Kodak X-ray film (Biomax MR) at -70 °C.

Primer extension. Primer extension was performed using the L358P27 primer. Ten picomoles of the non-phosphorylated primer was labelled with T4 polynucleotide kinase (Promega) in the presence of [γ -³²P]ATP. RNA (6 μ g) and the labelled primer (0.1 pmol) were mixed in a volume of 7 μ l containing 50 mM Tris/HCl (pH 8.3) and 0.1 M KCl. The reaction was then incubated for 1 min at 94 °C, 10 min at 55 °C and 15 min on ice. The volume of the mixture was adjusted to 12 μ l by the

addition of 1 μ l of a mixture containing dNTPs (2.5 mM each), 0.5 μ l RNasin (Promega), 2.2 μ l reverse transcriptase 5 $\times$ buffer (0.25 M Tris/HCl, pH 8.3; 0.2 M KCl; 36 mM magnesium acetate, 0.01 M DTT), 0.8 μ l diethylpyrocarbonate-treated water and 0.5 μ l avian myeloblastosis virus reverse transcriptase (RT; Promega). Reverse transcription was performed at 42 °C for 45 min and stopped by addition of 5 μ l stop buffer.

Samples were electrophoresed in a 6% polyacrylamide gel containing 8 M urea alongside the sequencing products obtained with the same oligonucleotide primer. Gels were fixed in 5% (v/v) methanol/5% (v/v) acetic acid, and exposed to X-ray film (Kodak X-Omat RS) for 24 h at -70 °C.

RT-PCR. Synthesis of the first strand of cDNA was performed using 3 μ g total *M. bovis* BCG RNA as template and random hexamers as primers, following the indications in the 'SUPERScript Preamplification System for First Strand cDNA Synthesis' kit (Life Technologies). Ribonuclease-treated RNA samples were used as a negative control. A 5 μ l aliquot of the cDNA synthesis reaction was amplified with primers 2-1Dir/2-1Av4 and 2-1Dir/2-1Rev. Amplification conditions were denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Amplification products were detected in 1% agarose gels. The specificity of the amplified bands was determined by Southern blotting using [α -³²P]dCTP pMBA21 as probe.

SDS-PAGE and Western blotting. Cells were harvested by centrifugation, washed and resuspended in PBS buffer, and lysed by boiling in loading buffer (2% SDS; 0.125 M Tris/HCl, pH 6.8; 1% 2-mercaptoethanol; 0.02% bromophenol blue; 10% glycerol) for 10 min. Proteins (50 μ g) were separated by electrophoresis in 15% SDS-PAGE gels by the technique of Laemmli (1970), and electrotransferred onto nitrocellulose using a Bio-Rad Trans-Blot Cell tank transfer unit at 150 mA for 2 h in 25 mM Tris/HCl (pH 8.0), 0.19 M glycine and 20% (v/v) methanol. Transfer yield was visualized by transient staining with Ponceau Rouge. Non-specific sites in the blot were blocked by incubation for 1 h with 5% dried non-fat powdered milk in 20 mM Tris/HCl (pH 7.5), 0.5 M NaCl buffer (TBS) at room temperature. Nitrocellulose membranes were incubated overnight at 4 °C with a 1:300 dilution of anti-P27 polyclonal serum followed by an alkaline-phosphatase-conjugated secondary antibody for 2 h at room temperature. Colour reaction was developed for 30 min by the addition of 5-bromo-4-chloro-3-indolyl phosphate and toluidinium nitro blue tetrazolium as substrates.

RESULTS

Determination of the transcription initiation point

The start point of P27 transcription was determined based on the primer extension assay using total *M. bovis* BCG RNA treated with DNase. An antisense primer whose 5' end corresponds to the nucleotide -25 relative to the A in the start codon was used. This reaction yielded two products, a larger one (P1), with a weaker signal consistent with a start at the A situated at 87 nt from the start codon, and a shorter product (P2), with the stronger signal, and corresponding to the most likely transcription start point, mapping to a G 76 nt upstream of the start codon (Fig. 1a, lane 2; Fig. 1b). However, the possibility that these extension products may result from a transcriptional stop of the reverse transcriptase in the assay due to RNA secondary structures cannot be

Fig. 1. Determination of transcriptional start points for the *P27* gene. (a) Primer extension reactions were carried out with end-labelled L358P27 primer annealing at nucleotide position –25 relative to the A residue of the initiation codon of the *P27* gene. This primer was then annealed to total RNA isolated from *M. bovis* BCG, and extended with reverse transcriptase. Reactions were analysed on urea-6% gels. Sequencing reactions were performed with the same oligonucleotide on pMBA21 and run alongside the primer extension reaction. Primer extension reactions were carried out in the presence (lane 1) or absence (lane 2) of ribonuclease ($20 \mu\text{g ml}^{-1}$). (b) Genetic arrangement upstream from the *P27* gene. The transcriptional start points (P1 and P2) are indicated; the –35 consensus sequence is boxed and the –10 consensus sequence is boxed and underlined. The essential promoting region is shaded; the putative Shine–Dalgarno sequence is underlined; the ATG translational start codon is indicated in bold.

excluded. Primer extension reactions were repeated three times, and the same banding pattern was observed. Ribonuclease treatment abolished the observed extension product (Fig. 1, lane 1).

P27 and *P55* genes are organized in an operon

The close proximity of an ORF encoding *P55* in the same transcriptional orientation downstream of the *P27* gene suggests that the *P27* and *P55* genes form an

operon. To investigate this possibility, Northern blot hybridization and RT-PCR reactions using total *M. bovis* BCG RNA were performed. For Northern blots, hybridizations were carried out using a specific radio-labelled antisense oligonucleotide probe, L358P27, which targets to nucleotide position –25 on the *P27* mRNA relative to the ATG start codon. A single band, heavier than 2 kb, was detected in *M. bovis* BCG RNA (Fig. 2a). The specificity of the oligonucleotide was demonstrated by the absence of detectable hybridization in total RNA from *M. smegmatis*. *M. bovis* BCG RNA treated with ribonuclease did not react, indicating that genomic DNA was not present. The possibility of cross-hybridization of the *P27* probe to the rRNA species was excluded by stripping the blot and rehybridizing with a 16S rRNA gene of *Bacillus subtilis*. RNA bands (one band of 1.5 kb for the small rRNA; and a weakly cross-reactive band of 3.2 kb for the large rRNA) showed different molecular masses than those observed with the *P27* probe (Fig. 2b). All samples were previously treated with DNases to avoid hybridization to DNA. Thus the *P27* mRNA transcript size is consistent with polycistronic transcription.

For RT-PCR assays, a population of cDNA was obtained by the use of a hexanucleotide mix in the retrotranscription reaction of *M. bovis* BCG RNA (treated with DNase), which was used in a second step as template in PCR reactions. Two combinations of primers were used for the amplification reaction: 2-1Dir/2-1Av4 and 2-1Dir/2-1Rev. See Fig. 2(d) for the position of each primer. RT-PCR produced amplification products of the expected size for polycistronic mRNA (1.617 kb with the primers 2-1Dir/2-1Rev and 898 bp with the primers 2-1Dir/2-1Av4) (Fig. 2c). The fidelity of the reaction was confirmed by Southern blotting using pMBA21 as a probe (data not shown). In ribonuclease-treated RNA samples no bands were observed in agarose gels or by Southern blotting, excluding the possibility that the products were amplified from genomic sequences.

Deletion analysis of the *P27* gene promoter

In order to map the *P27* gene promoter, deletion derivatives of the 5' non-coding region were generated. Four DNA fragments containing the *P27* gene plus different-sized DNA extensions (410, 161, 110 and 28 bp) of the 5' non-coding region were amplified by PCR and inserted into the pSUM41 vector. The resulting plasmids were named pMBA27, pMBA2741, pMBA2792 and pMBA27ΔP. All these constructions were introduced by electroporation into *M. smegmatis* mc²155. *P27* expression was assessed in cell extracts by Western blotting using an anti-*P27* serum (Fig. 3). *P27* expression was detected in *M. smegmatis*/pMBA27, *M. smegmatis*/pMBA2741 and *M. smegmatis*/pMBA2792 strains, while none was observed in *M. smegmatis*/pSUM41 and *M. smegmatis*/pMBA27ΔP (Fig. 3, lanes 1–5). To discard the possibility that the *plac* promoter of the pSUM vector drives *P27* gene transcription, a transcription terminator was added to the construction

Fig. 2. Analysis of the P27/P55 mRNA transcript. Total RNA (10 µg) extracted from *M. bovis* BCG (lanes 1 and 2) and *M. smegmatis* (lane 3) was separated on 0.8% agarose gels supplemented with formamide/formaldehyde, and processed for Northern blotting as described by Ausubel *et al.* (1996) in the presence (lane 1) or absence (lanes 2 and 3) of ribonuclease (20 µg ml⁻¹). Hybridization was carried out using either the L358P27 [³²P]ATP antisense oligonucleotide probe (a) or a radiolabelled 16S rRNA probe (b). Autoradiography was performed for 7 d or 24 h, respectively. (c) Total RNA (3 µg) from *M. bovis* BCG was analysed by RT-PCR. cDNA amplification was performed with random hexamer primers. (d) Primer positions of 2-1Dir/Av4 and 2-1Dir/2-1Rev used in PCR reactions.

carrying the minimal promoter region. The omega (Ω) interposon was cloned into the plasmid pMBA2792 downstream of the *plac* promoter, resulting in plasmid pMBA2792Ω. It has been demonstrated that insertion of

Fig. 3. Western blot of cell extracts from *M. smegmatis* clones carrying deletions of the 5' non-coding region of P27. Lanes: 1, *M. smegmatis*/pMBA27; 2, *M. smegmatis*/pMBA2741; 3, *M. smegmatis*/pMBA2792; 4, *M. smegmatis*/pMBA27ΔP; 5, *M. smegmatis*/pSUM41; 6, *M. smegmatis*/pMBA2792Ω; 7, *M. smegmatis*/pMBA28. The lanes were incubated with anti-P27 polyclonal serum.

Fig. 4. Determination of promoter activity of the 5' non-coding region in *M. smegmatis*. Genetic elements are indicated. The stem-loop indicates a possible transcriptional terminator. Plasmids harbouring different 5' non-coding deletions were obtained by cloning of PCR amplification products into pSUM41. Expression of the P27 recombinant protein is indicated.

an Ω fragment into DNA leads to termination of RNA synthesis in *M. smegmatis* (Timm *et al.*, 1994). In this study, P27 expression was detected in *M. smegmatis*/pMBA2792Ω (Fig. 3, lane 6). To test the integrity of the P27 gene in the shorter insert (that showing no expression), the P27 coding sequence in the pMBA27ΔP plasmid was subcloned downstream of the *hsp60* promoter into the pMV261 vector, to give the pMBA28 plasmid. P27 expression was observed in *M. smegmatis*/pMBA28 (Fig. 3, lane 7), confirming that the insert has a potentially functional gene.

Deletion analysis showed that a sequence important for promoter activity lay between 28 and 110 nt upstream of the start codon (Fig. 4). Analysis of the region upstream of transcriptional start point P2 revealed two potential –10 hexamers, one (GAACAT) 8 bp upstream which

has 3/6 matches with the typical *E. coli* -10 consensus sequence (TATAAT), 4/6 matches with the mycobacterial superoxide dismutase gene promoter (GATTAT) (Zhang *et al.*, 1991) and 4/6 matches with the promoter of a gene encoding the 38 kDa protein of *Mycobacterium tuberculosis* (GAAATT) (Andersen & Hansen, 1989), and another (CATCGC) 5 bp upstream of the transcription start point which has 4/6 matches with the -10 promoter sequence of the *mpb70* gene (CATCAG) (Matsuo *et al.*, 1995). In addition, we found a -35 hexamer positioned at 26 nt from the transcription start point which has 4/6 matches with the -35 consensus sequence of the *hsp60* promoter of *M. bovis* BCG (TTGCAC) (Shinnick, 1987; Thole *et al.*, 1987).

However, at 7 bp upstream of the longer extension product (P1) there is an AACAAAT motif that matches five of six positions of the -10 (AAGAAT) promoter sequence of the *hsp60* gene of *M. bovis* BCG. Around the corresponding -35 position, we identified several potential consensus sequences which are homologous to published promoter sequences of *M. bovis* and *M. tuberculosis*. However, these -35 elements are outside of the minimal promoting region as determined by the deletion experiment, indicating that they are not essential to the transcription of the *P27* gene under the culture conditions used in this study.

DISCUSSION

To study the expression and transcriptional features of the *P27* gene, we characterized its 5' non-coding region. We mapped the *P27* gene promoter region by the progressive 5' deletion of the putative regulatory sequence. Our results revealed that there is a minimal promoter region between bases -28 and -110 relative to the start codon. We defined this promoter region as an essential sequence to transcribe the *P27* gene under the culture conditions used in this work. Two possible transcription initiation points were identified, but only one (P2), which maps 76 bases upstream of the proposed ATG initiation codon, had associated potential promoter sequences placed in the minimal region with promoter activity. Probably, a second promoter (P1) is localized in the deleted region. Operons with more than one promoter have been described in mycobacteria which are active under different physiological conditions (Suzuki *et al.*, 1991; Movahedzadeh *et al.*, 1997; Verma *et al.*, 1999). It could be possible that the P1 promoter is dominant under other conditions and the promoter region may be dynamic depending on the growth conditions.

The recognition of promoter sequences in prokaryotes is mainly mediated by the σ subunit of RNA polymerase. In most bacteria, these promoters are constituted by hexamers placed in positions -10 and -35 or in their vicinity. In *E. coli*, the -10 and -35 consensus sequences and the distance that separates them have been extensively studied. However, this information is not directly applicable to *M. tuberculosis* or *M. bovis*,

because, in general, mycobacterial promoters function poorly in *E. coli*, indicating that mycobacteria may possess different transcriptional signals. Despite their importance as microbial pathogens and heterologous expression systems, little is known about the structure of mycobacterial gene promoters. Several promoters were predicted by either computer analysis or their distances from experimentally determined transcription start points, using conventional *E. coli* promoter spacing and sequences as a guideline, but only in some cases was promoter activity experimentally confirmed. The low homology among the promoter sequences reported makes the establishment of a specific consensus sequence for mycobacterial promoters difficult. However, a number of mycobacterial consensus sequences have been proposed. Bashyam *et al.* (1996) used mycobacterial promoter sequences isolated from *M. tuberculosis* and *M. smegmatis* to generate the following probable consensus sequence for the -10 region: T (100%), A (93%), T (50%), A (57%), a (43%) and T (71%) for *M. smegmatis* and T (80%), A (90%), Y (60%), g (40%), A (60%) and T (100%) for *M. tuberculosis*. However, they were unable to find a single strongly conserved sequence in the -35 regions. Using all the putative mycobacterial sequences published, Mulder *et al.* (1997) calculated the mycobacterial promoter consensus sequence as follows: -35: T (92%), T (53%), G (62%), A (44%), C (60%), G (31%)/A (26%); and -10: T (68%), A (76%), T (41%), A (36%)/G (26%), A (34%)/C (28%), T (76%). During analysis of the 5' untranslated *P27* gene region two overlapping putative -10 (GAACAT, CATCGC) sequences were identified. The GAACAT -10 hexamer proposed conserves the second and the last two positions on the Mulder *et al.* (1997) consensus. We also identified a putative -35 sequence (TTCCTC) which shares 66% identity with other mycobacterial promoters. Other putative promoters were described in our previous paper (Bigi *et al.*, 1997). However, these potential promoters as well as the -35 element associated with transcriptional start point P1 are outside the essential promoting region. The future characterization and mapping of the P2 promoter are necessary to understand the regulation mechanisms of *P27/P55* operon transcription. It is possible that the promoters are recognized by different RNA polymerase holoenzymes, and they are utilized to different extents during growth.

The spacing between the putative -10 and -35 regions is in agreement with other mycobacterial promoters reported. It has been demonstrated that the mycobacterial promoter can accommodate a large variety of sequences between the -35 and -10 regions. Such an analysis was performed by Kremer *et al.* (1995), who observed that the distances from 4 bp to 64 bp are functional in the 85A promoter.

Since the mapping of the *P27* gene promoter was performed in *M. smegmatis* for reasons of convenience, it would be important to confirm these results in a native producer species (*M. bovis* and *M. tuberculosis*). However, Bashyam *et al.* (1996) suggested that the basic

transcriptional machineries of *M. smegmatis* and *M. tuberculosis* have transcriptional specificity determinants in common. Thus *M. smegmatis* can be safely used as a surrogate host for expression of at least the constitutively expressed genes from slowly growing pathogenic mycobacteria.

The proximity of an ORF downstream of the *P27* gene led us to suppose the existence of a polycistronic messenger which would be the transcription product of both genes. Although polycistronic messengers are often found in prokaryotes, to our knowledge only a few mRNAs of this type have been identified in *M. bovis*, e.g. ESAT-6 antigen, which was found to be encoded in a transcriptional unit formed by two genes (Berthet *et al.*, 1998). Many more polycistronic transcripts should exist in *M. bovis* and *M. tuberculosis*, since analysis of the genome sequence of *M. tuberculosis* (Cole *et al.*, 1998) allows the identification of various potential operons. Using Northern blotting and RT-PCR assays, we demonstrated that *P27* and a gene encoding a putative antibiotic transporter (*P55*) belong to the same transcriptional unit. Such a genetic organization suggests that a functional relationship exists between the products of both genes. The search for the role of this operon in *M. bovis* is currently under way.

Interestingly, three putative membrane proteins from *M. tuberculosis*, *LprA*, *LprF* and *LppX* (accession nos Z77137, Z81011 and Z83858, respectively), share high homology with *P27*. The ORFs encoding these three proteins as well as the *P27* version of *M. tuberculosis* (*lprG*; accession no. Z80108) were identified during the *M. tuberculosis* genome sequencing project (Cole *et al.*, 1998) but in contrast to the *P27* gene, they are not adjacent to genes encoding drug transporters and do not seem to be organized in an operon-like structure. Immunoblot studies indicated that *P27* shares epitopes with *LprA* (data not shown). The function of *P27* and these related sequences is not known, but the close identity suggests that they may belong to a novel membrane antigenic protein family.

Future studies will help determine whether this conserved sequence reflects a similarity in cellular function of these proteins.

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REFERENCES

- Aínsa, J. A., Martín, M., Cabeza, M., De la Cruz, F. & Mendiola, M. V. (1996). Construction of a family of *Mycobacterium/Escherichia coli* shuttle vectors derived from pAL5000 and pACYC184: their use for cloning an antibiotic-resistance gene from *Mycobacterium fortuitum*. *Gene* **176**, 23–26.
- Andersen, A. B. & Hansen, E. B. (1989). Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular weight protein of *Mycobacterium tuberculosis*. *Infect Immun* **57**, 2481–2488.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1996). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bashyam, M. D., Kaushal, D., Dasgupta, S. K. & Tyagi, A. K. (1996). A study of the mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J Bacteriol* **178**, 4847–4853.
- Berthet, F.-X., Rasmussen, P. B., Rosenkrands, I., Andersen, P. & Gicquel, B. (1998). A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CPF-10). *Microbiology* **144**, 3195–3203.
- Bigi, F., Espitia, C., Alito, A., Zumarraga, M., Cravero, S. & Cataldi, A. (1997). A novel 27 kDa lipoprotein antigen from *Mycobacterium bovis*. *Microbiology* **143**, 3599–3605.
- Braibant, M., Lefevre, P., de Wit, L., Ooms, J., Peirs, P., Huygen, K., Wattiez, R. & Content, J. (1996a). Identification of a second *Mycobacterium tuberculosis* gene cluster encoding proteins of an ABC phosphate transporter. *FEBS Lett* **394**, 206–212.
- Braibant, M., Lefevre, P., de Wit, L., Peirs, P., Ooms, J., Huygen, K., Andersen, A. B. & Content, J. (1996b). A *Mycobacterium tuberculosis* gene cluster encoding proteins of a phosphate transporter homologous to the *Escherichia coli* Pst system. *Gene* **176**, 171–176.
- Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Dale, J. W. & Patki, A. (1990). Mycobacterial gene expression and regulation. In *Molecular Biology of the Mycobacteria*, pp. 172–189. Edited by J. McFadden. Guildford: Surrey University Press.
- DeMaio, J., Zhang, Y., Ko, C. & Bishai, W. R. (1997). *Mycobacterium tuberculosis* sigF is part of a gene cluster with similarities to the *Bacillus subtilis* sigF and sigB operons. *Tuber Lung Disc* **78**, 3–12.
- Harth, G., Lee, B. Y., Wang, J., Clemens, D. L. & Horwitz, M. A. (1996). Novel insights into the genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*. *Infect Immun* **64**, 3038–3047.
- Kremer, L., Baulard, A., Estaquier, J., Content, J., Capron, A. & Locht, C. (1995). Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. *J Bacteriol* **177**, 642–653.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Matsuo, T., Matsumoto, S., Ohara, N., Kitaura, H., Mizuno, A. & Yamada, T. (1995). Differential transcription of the MPB70 genes in two major groups of *Mycobacterium bovis* BCG substrains. *Microbiology* **141**, 1601–1607.
- Movahedzadeh, F., Colston, M. J. & Davis, E. O. (1997). Determination of DNA sequences required for regulated *Mycobacterium tuberculosis* RecA expression in response to DNA-damaging agents suggests that two modes of regulation exist. *J Bacteriol* **179**, 3509–3518.
- Mulder, M. A., Zappe, H. & Steyn, L. M. (1997). Mycobacterial promoters. *Tuber Lung Disc* **78**, 211–223.
- Papavinassasundaram, K. G., Movahedzadeh, F., Keer, J. T.,

- Stoker, N. G., Colston, M. J. & Davis, E. O. (1997).** Mycobacterial *recA* is cotranscribed with a potential regulatory gene called *recX*. *Mol Microbiol* **24**, 141–153.
- Prentki, P. & Krisch, M. (1984).** In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303–313.
- Shinnick, T. M. (1987).** The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J Bacteriol* **169**, 1080–1088.
- van Soolingen, D., Hermans, P. W. M., de Haas, P. E. W., Soll, D. R. & van Embden, J. D. A. (1991).** Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* **29**, 2578–2586.
- Stover, C. K., de la Cruz, V. F., Fuerst, T. R. & 11 other authors (1991).** New use of BCG for recombinant vaccines. *Nature* **351**, 456–460.
- Suzuki, Y., Nagata, A. & Yamada, T. (1991).** Analysis of the promoter region in the rRNA operon from *Mycobacterium bovis* BCG. *Antonie Leeuwenhoek* **60**, 7–11.
- Thole, J. E., Keulen, W. J., Kolk, A. H., Groothuis, D. G., Berwald, L. G., Tiesjema, R. H. & van Embden, J. D. (1987).** Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. *Infect Immun* **55**, 1466–1475.
- Timm, J., Lim, E. M. & Gicquel, B. (1994).** *Escherichia coli*-mycobacteria shuttle vectors for operon and gene fusion to lacZ: the pJEM series. *J Bacteriol* **176**, 6749–6753.
- Verma, A., Sampla, A. K. & Tyagi, J. S. (1999).** *Mycobacterium tuberculosis* rrn promoters: differential usage and growth rate-dependent control. *J Bacteriol* **181**, 4326–4333.
- Wards, B. J. & Collins, D. M. (1996).** Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. *FEMS Microbiol Lett* **145**, 101–105.
- Young, D. B., Kaufmann, S. H. E., Hermans, P. W. M. & Thole, J. E. R. (1992).** Mycobacterial protein antigens: a compilation. *Mol Microbiol* **6**, 133–145.
- Zhang, Y., Lathigra, R., Garbe, T., Catty, D. & Young, D. (1991).** Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis*. *Mol Microbiol* **5**, 381–391.

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