Activity of mycobacterial promoters during intracellular and extracellular growth

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pUS933, a bifunctional *Mycobacterium*–*Escherichia coli* translational fusion vector containing an amino-terminally truncated *E. coli lacZ* reporter gene, was constructed. Derivatives of pUS933, containing the promoter, RBS and start codon of the *Mycobacterium bovis* BCG *hsp60* gene, the *Mycobacterium leprae* 28 kDa gene and the *M. leprae* 18 kDa gene were constructed and introduced into *E. coli*, *Mycobacterium smegmatis* and *M. bovis* BCG. β -Galactosidase activity was measured for mycobacteria grown in liquid culture. Primer-extension analysis was used to determine the transcriptional start point for the 18 kDa promoter in *M. smegmatis*. Murine macrophages were infected with recombinant BCG containing the pUS933 derivatives and expression levels were examined, by fluorescence microscopy and fluorometry, during intracellular growth of BCG. Both the BCG *hsp60* gene promoter and the *M. leprae* 28 kDa gene promoter gave high levels of β -galactosidase expression in all situations examined. In contrast, the *M. leprae* 18 kDa promoter fragment gave very low levels of expression in *M. smegmatis* and BCG grown in liquid culture, but in BCG growing within macrophages it was induced to levels almost as high as the other promoters. This indicated that the 18 kDa gene is specifically activated during intracellular growth and may therefore be involved in survival of *M. leprae* within macrophages. This pattern of regulation may be useful for controlling expression of foreign genes in recombinant BCG strains.

Keywords: mycobacteria, promoter, gene expression, macrophage

INTRODUCTION

The mycobacteria are an important group of organisms which includes *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy. BCG (bacille Calmette–Guérin), an avirulent *Mycobacterium bovis* strain, is currently the world's most widely used vaccine, with a low incidence of serious complications. This and the extraordinary adjuvant properties of mycobacteria make BCG an attractive host strain for the development of recombinant vaccines (Bloom *et al.*, 1990; Stover *et al.*, 1991). During the last few years several vectors have been developed enabling cloning and expression of foreign genes in mycobacteria

(Snapper *et al.*, 1988; Husson *et al.*, 1990; Fuerst *et al.*, 1991; Stover *et al.*, 1991, 1993; Murray *et al.*, 1992; Dellagostin *et al.*, 1993). The development of expression systems in mycobacteria has been achieved by using sequences derived from the region upstream from characterized mycobacterial genes that are presumed to contain the active promoter. Genes that have been used to provide signals for expression of cloned genes in BCG include those encoding members of the conserved Hsp60 and Hsp70 families of stress proteins (Aldovini & Young, 1991; Stover *et al.*, 1991; Fuerst *et al.*, 1991; Connell *et al.*, 1993; Yasutomi *et al.*, 1993), the secreted α -antigen from *M. kansasii* (Matsuo *et al.*, 1990), a promoter region from *Mycobacterium paratuberculosis* named P_{AN} (Murray *et al.*, 1992), the promoter of the *groES/groEL1* operon of *Streptomyces albus* (Winter *et al.*, 1991), the gene encoding the *M. leprae* 18 kDa antigen (Dellagostin *et al.*, 1993), and the sequences encoding the 19 kDa lipoprotein promoter and signal peptide from *M. tuberculosis* (Stover *et al.*, 1993).

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Abbreviations: BCG, bacille Calmette–Guérin; FDG, fluorescein-di- β -galactopyranoside.

Expression of recombinant proteins has been demonstrated *in vitro* and transcriptional start sites have been mapped for a small number of genes (Stover *et al.*, 1991; Murray *et al.*, 1992; Levin & Hatfull, 1993). However, nothing is known about the *in vivo* level of expression of these genes. Since BCG is given as a live vaccine, expression of recombinant genes within the parasitized macrophage is likely to be critical for the subsequent immune response.

In this study we describe the development and use of a system that enables assessment of promoter activity during growth inside macrophage cells and we demonstrate the use of this system to analyse the promoter activity of previously characterized mycobacterial gene sequences and to identify new promoters.

METHODS

Bacterial strains and plasmids. The Glaxo strain of BCG Pasteur was used as a source of *M. bovis* BCG DNA. *Escherichia coli* DH5 α , *M. smegmatis* mc²155 (Snapper *et al.*, 1990) and BCG Pasteur were used for characterization of promoter activity. *M. bovis* BCG was grown in Middlebrook 7H9 broth (Difco) or Middlebrook 7H11 agar (Difco) supplemented with oleic acid albumin dextrose (OADC; Difco), 0.2% glycerol and 0.05% Tween 80 (Sigma). *E. coli* was grown in Nutrient Broth no. 2 (NB) (Oxoid). For solid media, 1% (w/v) bacteriological agar (Oxoid) was added. *M. smegmatis* was grown in the same medium as for *E. coli* with the addition of 0.2% glycerol and 0.05% Tween 80. When necessary the medium was supplemented with 15 μ g kanamycin ml⁻¹ for plasmid selection and 40 μ g X-Gal ml⁻¹ for identification of recombinant clones with promoter activity. Plasmid pUC18 was obtained from BRL. The plasmid pAL5000 (Labidi *et al.*, 1985a, b; Rauzier *et al.*, 1988) was extracted from *M. fortuitum* and digested with *EcoRV*.

Enzymes. Restriction enzymes, *Taq* DNA polymerase and calf intestinal alkaline phosphatase were from Boehringer Mannheim. The Klenow fragment of *E. coli* DNA polymerase I, T4 polynucleotide kinase and T4 DNA ligase were from BRL.

Construction of the promoter probe vector. The promoterless *E. coli lacZ* gene was obtained by *Bam*HI digestion of pMC1871 (Shapira *et al.*, 1983). It lacks an RBS and the first eight non-essential N-terminal codons. The *Bam*HI fragment was cloned into the *Bam*HI site of pUC18 to give pUS928. The *Bam*HI fragment from pUC4K (Taylor & Rose, 1988), containing the kanamycin-resistance gene from Tn903, was blunt-ended with the Klenow fragment of DNA polymerase I and ligated to *Sma*I-digested pUS928 to give pUS929. To provide a mycobacterial origin of replication, pUS929 was digested with *Sca*I and ligated to *EcoRV*-digested pAL5000 (Labidi *et al.*, 1985a, b, 1992; Rauzier *et al.*, 1988; Ranes *et al.*, 1990) to generate pUS933 (Fig. 1) which can replicate in *E. coli* and mycobacteria.

Cloning of characterized promoters. The *hsp60* gene promoter from *M. bovis* BCG was amplified by PCR using BCG DNA as template and with the primers PI (5'-TGCGCGatGcGACCA-CAACG-3') and PII (5'-GTACGCAatgcGTCTTGCCA-3') (*Sph*I site underlined, mismatched bases in lower case). The 404 bp PCR product was digested with *Sph*I and ligated into the *Sph*I site of pUS933 to generate pUS934. The *M. leprae* 18 kDa gene Large promoter (including 256 bp of regulatory sequence) was obtained by *EcoRI* digestion of pUS931 (Dellagostin *et al.*, 1993) and the 18 kDa gene Small promoter (including only 136 bp of regulatory sequence) was similarly obtained by *EcoRI*

digestion of pML3 (Booth *et al.*, 1988). Both DNA fragments were blunt-ended by Klenow fragment of DNA polymerase I. The vector pUS933 was digested with *Sph*I, trimmed back with T4 DNA polymerase and the 18 kDa gene promoters were separately cloned into it to generate pUS935 and pUS936. The 28 kDa gene promoter from *M. leprae* was generated by PCR using *M. leprae* genomic DNA as template. Primer 1: 5'-GTCATCGGTTGCCCTAGAGTA-3'; primer 2: 5'-CTTGC-ATCGGCGTCGGTTTCG 3'. The PCR product was blunt-end ligated into the *Sma*I site of pMC1871 (Shapira *et al.*, 1983). The resulting plasmid was digested with *Sa*I and the fragment containing the 28 kDa gene promoter and the *lacZ* gene was excised, blunt-ended and ligated into the *EcoRV* site of pYUB12 (Snapper *et al.*, 1988) to give pUS170.

Isolation of RNA. RNA isolation from *M. smegmatis* grown under standard conditions in liquid media, was performed by using a RNA extraction kit (Pharmacia) according to the manufacturer's instructions. Briefly, *M. smegmatis* was grown in 50 ml NB and harvested by centrifugation. The pellet was resuspended in 3 ml extraction buffer (buffered aqueous solution containing guanidinium thiocyanate, N-lauryl sarcosine, and EDTA) and the cells were lysed by sonication. Insoluble debris was removed by centrifugation at 5000 g for 20 min and the supernatant was loaded on top of a 2.7 ml cushion of caesium trifluoroacetate and centrifuged at 35000 r.p.m. in a SW50.1 rotor for 12 h at 15 °C. The RNA pellet was dissolved in water and stored at -80 °C as a propan-2-ol precipitate.

Primer-extension analysis. The oligonucleotide primer 5'-ACGGAACGGGTCAGTACGCAT-3', which is complementary to the beginning of the coding sequence of the 18 kDa gene (Booth *et al.*, 1988), was end-labelled with [γ -³²P]ATP (6000 Ci mmol⁻¹; 222 TBq mmol⁻¹) using T4 polynucleotide kinase. The radiolabelled primer was purified by chromatography on a Sep-Pak C₁₈ (Millipore) column, and the primer-extension reaction was performed as previously described (Sambrook *et al.*, 1989). Products of the primer-extension reaction were separated on a 6% (w/v) polyacrylamide/urea gel and visualized by autoradiography. Both strands of the DNA were sequenced with the Sequenase version 2.0 kit (USB) and the same primer.

Transformation of mycobacteria. *M. smegmatis* mc²155 cells for electroporation were prepared as described by Snapper *et al.* (1990), with a few modifications. Briefly, 50 ml NB supplemented with 0.05% Tween 80 (Sigma) and 0.2% glycerol in a 250 ml flask was inoculated with 0.5 ml of a saturated culture and incubated on a shaker for 48 h at 37 °C. After incubating on ice for 30 min, cells were harvested by centrifugation at 4000 g for 10 min, the pellet was resuspended and washed in 25 ml ice cold 1 mM HEPES, pH 7.0 and then re-centrifuged. After washing the pellet with 10 ml 10% (v/v) glycerol, it was resuspended in 2 ml 10% glycerol and stored at -70 °C in 400 μ l aliquots. Aliquots (100 μ l) of cells were mixed with 0.05–0.2 μ g DNA and transformed in 0.2 cm electroporation cuvettes, using the BioRad Gene Pulser set at 2.5 kV, 25 μ F and 600 Ω . The cells were immediately diluted in 0.9 ml NB, transferred to a polypropylene tube (Falcon 2059) and incubated at 37 °C for 2 h before plating on 7H11 medium containing 15 μ g kanamycin ml⁻¹. Antibiotic-resistant transformants were selected after 3 d of growth at 37 °C.

For BCG transformation, 7H9 or 7H11 medium supplemented with OADC, Tween 80 and glycerol were used. Cells for electroporation were inoculated into 500 ml tissue culture flasks containing 80 ml medium and incubated at 37 °C for 4 d. Cells were prepared for electroporation as described by Snapper *et al.* (1988), and after electroporation the cells were shaken for 4 h at 37 °C and then plated and incubated at 37 °C for 3 weeks.

β -Galactosidase assay using ONPG. β -Galactosidase was assayed in *E. coli* and mycobacteria as described by Miller (1992): a 0.1 ml volume of a late-exponential phase culture (OD_{600} 0.8–1.2) was added to 0.9 ml Z buffer (6 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM $MgSO_4$, 50 mM β -mercaptoethanol) and permeabilized with 20 μ l chloroform and 10 μ l 0.1% SDS. After vortexing for 10 s and 5 min incubation at 28 °C, 0.2 ml 4 mg ONPG ml^{-1} (Sigma) was added and the incubation was continued at 28 °C. When sufficient yellow colour had developed, the reaction was stopped by adding 0.5 ml 1 M Na_2CO_3 and the incubation time was recorded. After centrifuging for 2 min at 10 000 g , the OD_{420} was measured and β -galactosidase activity in Miller units was calculated by the formula: $(OD_{420} \times 1000)/(t \times v \times OD_{600})$, where t is the incubation time in minutes and v is the volume in ml of culture used.

Tissue culture. The mouse macrophage cell line J-774 was grown in RPMI 1640 (Sigma) containing 10% (v/v) foetal calf serum and 2 mM glutamine. Five millilitres of a suspension of 5×10^5 viable cells ml^{-1} were used to inoculate a 25 cm^2 tissue culture flask, followed by overnight incubation at 37 °C in 5% CO_2 .

Infection of monolayer. Bacterial cultures were diluted directly into fresh tissue culture medium to achieve a rate of infection of about 50 bacteria per macrophage. This inoculating medium was used to replace the growth medium and phagocytosis was allowed to occur for 4 h at 37 °C and 5% CO_2 . The monolayers were then washed twice with Hank's salt solution (Sigma) to remove non-phagocytosed bacteria, and returned to the incubator with fresh medium containing 50 μ g gentamicin ml^{-1} (Sigma) for 24 h, to kill any extracellular bacteria remaining after the washes.

Assay of β -galactosidase activity in infected macrophages.

The β -galactosidase activity in macrophages infected with recombinant mycobacteria expressing the *lacZ* gene from different promoters was detected by using the FluoroReporter *LacZ* Detection Kit (Molecular Probes) according to the manufacturer's instructions. Briefly, the infected macrophage cells were removed from the tissue culture flask by trypsin treatment and resuspended in staining medium [PBS (0.145 M NaCl, 0.15 M sodium phosphate), 4% foetal calf serum, 10 mM HEPES, pH 7.2] containing 300 μ M chloroquine (an inhibitor of the endogenous β -galactosidase) at 10^7 cells ml^{-1} . Cells (100 μ l) were transferred to a flow cytometer tube and placed in a 37 °C water bath for 20 min. The substrate, 2 mM fluorescein-di- β -galactopyranoside (FDG; Molecular Probes) in water, was loaded by adding 100 μ l pre-warmed (37 °C) solution to the 100 μ l of cells. After exactly 1 min at 37 °C, the FDG loading was stopped by adding 1.8 ml ice-cold staining medium and the cells were kept on ice. After 10 min, 40 μ l 50 mM phenethyl- β -D-thiogalactopyranoside was added to stop the reaction and the cells were analysed by using a flow cytometer FACScan, equipped with an argon ion laser and FACScan research software (Becton Dickinson).

RESULTS

Construction of the vectors

The translation fusion vector pUS933 (Fig. 1) containing a truncated *E. coli lacZ* gene without promoter or RBS, was constructed to test mycobacterial expression signals. The *E. coli lacZ* gene has already been successfully used as a reporter gene in mycobacteria (Barletta *et al.*, 1992) allowing the identification of recombinant clones which

Fig. 1. (a) Map of the promoter probe vector pUS933. The *lacZ* reporter gene, the pUC18 *lacZ* α fragment with its promoter (*Plac*) and the pUC18 β -lactamase (*bla*) are shown. The construction is described in the text. The constituent segments from different sources are distinguished by different shading. (b) Sequence around the cloning site (arrow indicates translational reading frame).

express enzymically active β -galactosidase fusion proteins. The kanamycin resistance gene from Tn903 was used as a selectable marker and the pAL5000 plasmid (Labidi *et al.*, 1985a, b, 1992; Rauzier *et al.*, 1988) provided the mycobacterial origin of replication to generate pUS933. This vector contains two unique restriction sites (*SphI* and *XbaI*) upstream of the promoterless *lacZ* gene that are useful for cloning expression signals. Negligible β -galactosidase activity (less than 2 Miller units) was detected with the vector alone, indicating that promoters present in the vector do not drive significant levels of β -galactosidase expression. Recombinant plasmids were constructed (Fig. 1) in which this reporter gene was driven by upstream sequences from previously characterized mycobacterial genes, namely the BCG *hsp60* gene (Thole *et al.*, 1987), the *M. leprae* 28 kDa antigen-encoding gene which is believed to be iron regulated (Cherayil & Young, 1988; Dale & Patki, 1990) and the *M. leprae*

Table 1. Promoter fragments examined

Plasmid	Promoter fragment
pUS934	0.4 kb fragment containing 383 bp of 5' regulatory region plus the first six codons from the BCG <i>hsp60</i> gene
pUS935	0.34 kb fragment containing 256 bp of 5' regulatory region plus the first 29 codons of the <i>M. leprae</i> 18 kDa promoter
pUS936	0.27 kb fragment containing 136 bp of 5' regulatory region plus the first 29 codons of the <i>M. leprae</i> 18 kDa promoter
pUS170	0.3 kb fragment containing 276 bp of the 5' regulatory region plus the first eight codons of the <i>M. leprae</i> 28 kDa antigen gene

18 kDa antigen-encoding gene (Booth *et al.*, 1988). Transcriptional start points were known only for the *hsp60* gene which in BCG utilizes two transcriptional start sites at positions 183 and 216 bp (tsA and TsB, Fig. 3) upstream of the ATG translational start site (Stover *et al.*, 1991). Since no promoter sequence had been identified for the *M. leprae* 18 kDa antigen gene (Booth *et al.*, 1988), two fragments containing either 256 bp (18 kDa Large promoter) or 136 bp (18 kDa Small promoter) upstream of the ATG start codon, were examined. Fragments containing these upstream sequences and codons for the first amino acids of the proteins were cloned in-frame with the *lacZ* gene, producing plasmids pUS934, pUS935, pUS936 and pUS170 (Table 1).

Promoter activity during *in vitro* growth

pUS934, pUS935, pUS936 and pUS170 were used to transform *E. coli*, *M. smegmatis* and BCG and β -galactosidase activity was measured in cell extracts. pUS933 is a translational fusion vector and therefore the β -galactosidase activity obtained is a measure of transcription

initiation, promoter strength and the efficiency of the ribosome-binding site. A comparison of expression levels in these species is shown in Table 2. Since the expression in the different hosts may be affected by plasmid copy number, only relative differences between the individual clones are significant. The *hsp60* gene promoter gave the highest levels of expression both in *M. smegmatis* and BCG. Surprisingly, the 18 kDa promoter fragments (Large and Small) were relatively strong in *E. coli* but showed a very low level of expression in *M. smegmatis* and BCG compared with the 28 kDa gene promoter and the *hsp60* gene promoter. The 18 kDa Large promoter which contains 256 bp of upstream sequence shows a significantly higher level of expression than the 18 kDa Small promoter which contains only 136 bp of upstream sequence. This difference in activity was consistent in *E. coli*, *M. smegmatis* and BCG. None of the promoters showed any significant difference in the level of expression in *M. smegmatis* and BCG.

β -Galactosidase expression in BCG during growth in macrophages

The recombinant BCG strains were used to infect murine macrophages and the β -galactosidase activity during intracellular growth was determined. To measure this activity, it was necessary to incubate infected cells with a β -galactosidase substrate capable of penetrating both the macrophage cell membrane and the mycobacterial cell wall. The fluorescent substrate FDG has previously been shown to be capable of penetrating live mammalian cells in tissue culture (Ikenaka *et al.*, 1990). Preliminary experiments (not shown) demonstrated that FDG was also capable of staining live BCG cells expressing β -galactosidase constructs.

Monolayers of the murine macrophage cell line J-774 (Snyderman *et al.*, 1977) were infected with mycobacteria. After allowing 4 h for phagocytosis, the cells were rinsed to remove extracellular bacilli. Ziehl-Neelsen staining confirmed the absence of extracellular bacteria and

Table 2. Comparison of the activities of mycobacterial promoters

	β -Galactosidase activity				
	Shaken liquid culture (Miller units)			Intracellular bacteria (arbitrary fluorescence units)	Activation*
	<i>E. coli</i>	<i>M. smegmatis</i>	BCG		
				BCG	
pUS933 (vector)	0.6 (0.1)	1.6 (0.2)	0.2 (0.5)	0	0
pUS170 (28 kDa)	152 (25)	181 (38)	155 (18)	88 (18)	0.56
pUS934 (<i>hsp60</i>)	136 (26)	335 (39)	253 (34)	156 (24)	0.61
pUS935 18 kDa - L	176 (24)	20 (3)	15 (1.5)	75 (10)	5
pUS936 18 kDa - S	38 (5)	6 (1.3)	3.8 (0.5)	24 (11)	6.3

SD are shown in parentheses.

*Activation is the ratio of β -galactosidase activity in intracellular BCG (fluorescence units) to that in BCG in liquid culture (Miller units).

Fig. 2. Intracellular expression of mycobacterial promoters. (a) Ziehl-Neelsen staining of macrophage monolayers infected with recombinant BCG strain. Acid-fast mycobacteria are stained red and the macrophage nuclei are stained blue. (b) Macrophage infected with BCG expressing the β -galactosidase gene under the control of the *hsp60* promoter. Cells were incubated with FDG substrate and photographed with a fluorescent microscope. Mycobacteria expressing the reporter gene appear as yellow filaments. Bars, 25 μ m.

revealed that 75–80 % of macrophages were infected (Fig. 2a). Counting of mycobacteria within macrophages revealed that the numbers of mycobacteria per cell varied from 5 to more than 50. This range was similar for each of the BCG strains tested and for each experiment. Macrophages were incubated with gentamicin to kill extracellular bacteria, then incubated with FDG and examined by fluorescent microscopy. Intracellular mycobacteria were clearly visible as brightly staining fine rods within the macrophages infected with the recombinant BCG strains (Fig. 2b). No fluorescence was seen with macrophages similarly infected with control BCG cells.

A total of 10^4 cells from each sample were analysed by flow cytometry. The same number of macrophages infected with non-recombinant BCG were also analysed. The relative intensity of fluorescence obtained with control BCG was subtracted from that obtained with the recombinant BCG to give a value for β -galactosidase activity within the infected macrophages. The means of the results of three separate experiments are shown in Table 2. It is not possible to directly convert between the units of β -galactosidase activity obtained for extracellular growth (Miller units), with the arbitrary units of fluorescence measured by fluorometry for intracellular activity. To compare intracellular and extracellular expression, we therefore derived a value for relative intracellular activation (in arbitrary units) by dividing the value obtained for intracellular expression (units of fluorescence) by the value (Miller units) for extracellular activity (Table 2). The two *M. leprae* 18 kDa promoter fragments showed the highest relative intracellular activation, i.e. tenfold higher than the activation of the *hsp60* gene.

Identification of the *M. leprae* 18 kDa gene promoter

The *M. leprae* 18 kDa large promoter fragment consistently gave higher levels of β -galactosidase activity both *in vitro* and during intracellular growth. This suggested that the large fragment may contain an additional transcriptional start site, as has been reported for the *hsp60* promoter (Stover *et al.*, 1991). Since the upstream region of the 18 kDa gene promoter contains a number of possible *E. coli* σ^{70} consensus transcription initiation sites, prime-extension analysis was done to identify the promoter region. RNA was extracted from *M. smegmatis* transformed with either pUS935 or pUS936. A single transcriptional start point located 65 bp upstream of the 18 kDa gene start codon was found for both clones (Fig. 3a). Upstream of the transcriptional start point are –10 and –35 sequences that are similar to consensus *E. coli* σ^{70} consensus promoter sequences (Fig. 3b). For comparison, Fig. 3 also shows mycobacterial σ^{70} and heat shock consensus promoter regions that have been identified by location of transcription start sites.

DISCUSSION

We have established conditions for examination of mycobacterial promoter activity in mycobacteria grown *in vitro* and in mycobacteria replicating within macrophages. In this system, a promoter, an RBS and a start codon in the correct reading frame must be provided by the cloned DNA. A similar system has been used to isolate promoters from mycobacteriophages (Barletta *et al.*, 1992), and *M. tuberculosis* DNA (Das Gupta *et al.*, 1993; Timm *et al.*, 1994). Since the consensus sequences and the strengths of mycobacterial RBSs are not fully characterized, we used a

Fig. 3. Primer-extension analysis of the *M. leprae* 18 kDa gene promoter. (a) RNA was extracted from *M. smegmatis* transformed with pU935 (1) and pU936 (2). Analysis was done using a primer located just upstream of the ATG start codon. A single transcriptional start point, the central base of the triplet TAT, is located 65 bp upstream of the 18 kDa gene translational start codon. (b) DNA sequence of the *M. leprae* 18 kDa gene promoter indicating the transcriptional start site (*). Putative -10 and -35 regions, similar to *E. coli* σ^{70} consensus promoter sequences ($T_{82}T_{84}G_{78}A_{65}C_{54}A_{45}$ <15-21 bp> $T_{80}A_{95}T_{45}A_{60}A_{50}T_{96}$ <4-8 bp> *, where percentage base preferences at each position are subscripted; Hawley & McClure, 1983) and the RBSs are underlined. For comparison, the promoter regions of the *M. leprae* 28 kDa promoter, the *hsp60* gene and the P_{AN} promoter from *M. paratuberculosis* are also shown. Transcriptional start sites (*) have been located for the *hsp60* gene (Stover *et al.*, 1991; Levin & Hatfull, 1993), the P_{AN} promoter (Murray *et al.*, 1992) and the 18 kDa gene (this study). The *hsp60* gene utilizes two transcriptional start sites in BCG (Stover *et al.*, 1991), tsA (183 bp upstream of the ATG translational start codon: initiation produces two products that differ by one nucleotide) and tsB (216 bp upstream), although only tsA is recognized in *M. smegmatis* (Levin & Hatfull, 1993). Upstream of tsB are sequences similar to the -10 and -35 *E. coli* heat-shock consensus promoter sequences and these are shown together with the consensus.

translational rather than a transcriptional probe previously used to study mycobacterial promoters (Kieser *et al.*, 1986; Sirakava *et al.*, 1989; Thomas *et al.*, 1992).

The *hsp60* and other mycobacterial promoters have been shown to express antigens that induce an immune response in animal models (de la Cruz *et al.*, 1991; Stover *et al.*, 1991; Murray *et al.*, 1992). However, no studies have previously been done to determine the promoter strength during intracellular growth. Additionally, since the *hsp60* promoter gives high levels of expression during *in vitro* growth, it may be difficult to use this system to clone genes that are toxic to *in vitro*-grown mycobacteria. For example, attempts to clone the HIV *env* gene under the control of the *hsp60* promoter using a mycobacterial autonomously replicating plasmid vector, were unsuccessful, suggesting that expression of this gene was toxic to BCG (Stover *et al.*, 1991). For the development of a recombinant vaccine it would be useful to have a mycobacterial promoter that is expressed at low levels *in vitro* but is specifically activated during growth inside macrophages – the environment found by the bacteria once the vaccine is administered. The promoter of the *M. leprae* 18 kDa antigen gene appears to be specifically activated in this way and may therefore be useful in providing expression signals for driving foreign gene expression in recombinant BCG vaccines.

The intracellular up-regulation found for the 18 kDa gene is consistent with the known characteristic of the protein as a major antigen in natural infection. The *M. leprae* 18 kDa antigen is related to the alpha-crystallin family of low molecular mass heat-shock proteins of unknown function. Homologues of this family of proteins are found

in *M. tuberculosis* (Verbon *et al.*, 1992) and other mycobacteria (Booth *et al.*, 1993). The production of a homologous protein found in *Mycobacterium habana* was found to increase in response to heat shock (Lamb *et al.*, 1990); however this was not found for the *M. tuberculosis* homologue (Verbon *et al.*, 1992). We demonstrated that in *M. smegmatis*, the *M. leprae* 18 kDa gene utilizes a single transcriptional start point located 66 bp upstream of the start codon. Immediately upstream of the transcriptional start point are putative -10 and -35 sequences that are similar to *E. coli* σ^{70} consensus promoter sequences, indicating that in *M. smegmatis* during growth in liquid culture, the 18 kDa gene was transcribed by RNA polymerase associated with the major vegetative sigma factor of *M. smegmatis*. However, the region of the 18 kDa gene promoter (CTATATC) containing the putative -10 sequence, when aligned with the *E. coli* -10 hexamer consensus sequence (TATAAT), shows a mismatch at either the first or the last T residue, both of which are highly conserved in *E. coli* (Fig. 3c). An alternative interpretation is that the functional 18 kDa -10 sequence is a pentamer (TATAT). Since little is known of mycobacterial promoter structure, it is impossible to assess the significance of this finding. Further studies involving site-directed mutagenesis will be done to identify critical bases within the promoter region. Both the large and the small upstream 'promoter' fragments used in the reporter probe experiments were shown to utilize the same transcriptional start point, and therefore the difference between the level of expression found with these fragments cannot be due to the use of an additional transcriptional start point in the large fragment. A third recognition element, called the UP element, and a region adjacent to the UP element that contains binding sites for

the activator protein Fis have been found to extend some *E. coli* promoter regions to 150 bp upstream (Ross *et al.*, 1993).

The system described here will allow assessment of intracellular expression of genes from both *M. leprae*, *M. tuberculosis* and other mycobacterial pathogens. Genes that are selectively expressed during intracellular growth may be involved in intracellular survival and virulence of *M. tuberculosis* and *M. leprae*. Their identification may therefore provide new targets for chemotherapy, immunotherapy and/or prophylaxis.

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