Negative transcriptional regulation of the *mce3* operon in *Mycobacterium tuberculosis*

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***mce3* is one of the four *mce* operons in *Mycobacterium tuberculosis* that encode exported proteins with a probable role in the virulence of this bacterium. Upstream of *mce3* there is a putative regulatory gene (*Rv1963*) that harbours a double *tetR*-family signature. To study the role of this putative regulatory gene in the transcriptional regulation of the *mce3* operon, *Mycobacterium smegmatis* mc²155 and *M. tuberculosis* H37Rv strains that harboured gene fusions between the *mce3* promoter region and the *Escherichia coli* *lacZ* gene, either containing or not containing the *Rv1963* gene, were used. The presence of the *Rv1963* gene in the strains greatly reduced β -galactosidase activity, suggesting that the *Rv1963*-encoded protein is a transcriptional repressor of the *mce3* operon. Expression of *mce3* by recombinant *M. tuberculosis* was increased when it was grown in a macrophage-like cell line (J774), compared to the level of expression seen when the recombinant bacterium was grown under *in vitro* conditions. However, no lifting of repression was induced. The *mce3* promoter was defined by deletion and cloning of the *Rv1963*–*Rv1964* intergenic region in a 200 bp DNA fragment harbouring the region upstream of the *Rv1964* start codon. Gel-shift experiments determined that the *Rv1963*-binding site was located in this region. These results indicate that the *mce3* operon is transcriptionally regulated and that under certain, unknown, conditions repression of gene expression could be lifted.**

Keywords: repressor, *tetR*, reporter

INTRODUCTION

Tuberculosis (TB), a chronic illness caused by *Mycobacterium tuberculosis*, is still a major disease worldwide. According to the World Health Organization, TB is a considerable public health problem in Latin America, Asia and Africa. During the last few years, an increase in the incidence of TB has been observed and this has been attributed to weak control programmes, the AIDS pandemic, which predisposes individuals to developing TB, and the appearance of *M. tuberculosis* strains that are resistant to first-line antibiotics (Murray *et al.*, 1990).

Unfortunately, many aspects of the infection and disease

process of tuberculosis at the cellular and molecular level still remain unknown. According to Smith (1984), to infect and cause disease pathogenic mycobacteria must be able to (i) colonize the host's lung tissues, (ii) enter the host's cells, (iii) multiply in the environment of the host's tissues, (iv) resist or interfere with the host's defence mechanisms and (v) cause damage to the host's tissues. Despite the formidable advances in molecular biology in recent years, the identification of the virulence factors of pathogenic mycobacteria (i.e. *M. tuberculosis*, *Mycobacterium bovis* and *Mycobacterium leprae*) has been delayed, when compared to the identification of the virulence factors of other infectious bacteria. Factors contributing to this situation include the lack of a phenotype clearly associated with virulence in pathogenic mycobacteria and the scarcity of genetic tools for transforming and mutating *M. tuberculosis*. Steps to solving the latter problem have been taken, as improved

Abbreviations: *mce3R*, *Rv1963*; TSP, transcription start point; BAC, bacterial artificial chromosome.

genetic tools have allowed the identification of virulence-related genes in *M. tuberculosis* (Camacho *et al.*, 1999; Cox *et al.*, 1999). The most-employed virulence-associated attribute of *M. tuberculosis* is its persistence in the infected organism or in cultured cells (Bange *et al.*, 1999).

We have previously identified a 12.7 kb region in the genome of *M. tuberculosis* that is absent from the genome of *M. bovis* (Fisanotti *et al.*, 1997; Zumarraga *et al.*, 1999). This 12.7 kb fragment was present in all of the *M. tuberculosis* strains tested and was absent from all of the *M. bovis*, *Mycobacterium microti* and *Mycobacterium africanum* strains tested. The region is located near the 3' end of the RD2 element described by Mahairas *et al.* (1996), a 14 kb genomic locus present in *M. bovis* but absent from some strains of *M. bovis* BCG, suggesting that this region suffers from genetic instability. Sequence analysis of ORFs within the 12.7 kb fragment of the *M. tuberculosis* genome demonstrated that it mostly encodes exported proteins. One of the ORFs is highly homologous to the invasin-like protein described by Riley and colleagues (Arruda *et al.*, 1993; Chitale *et al.*, 2001). This region was also described by Gordon *et al.* (1999) as RD7. The presence of genes encoding an invasin-like protein and many membrane or secreted proteins within the 12.7 kb region suggests that this region may play an essential role in the host-pathogen interaction of *M. tuberculosis*. The ORFs within the region are organized as a putative operon, which is similar in its sequence and organization to three other *M. tuberculosis* regions (operons *mce1*, *mce2* and *mce4*) described by Cole *et al.* (1998). The lack of similarity of the genes encoded by this putative operon to genes from other bacterial species suggests that they may play a specific role in the physiology or virulence of *M. tuberculosis*. Flesselles *et al.* (1999) reported that a BCG strain mutated in *mce2* exhibits a reduced ability to invade the non-phagocytic epithelial cell line HeLa, which supports the idea of a role for *mce* operons in virulence. Harboe *et al.* (1999) demonstrated production of the *mce1*-encoded proteins using cell extracts from *M. tuberculosis* and *M. bovis* BCG. Mce1 proteins are recognized by the antibodies of TB patients (Ahmad *et al.*, 1999), indicating *in vivo* expression of the *mce1* operon. At the genetic level, the four *mce* operons appear to have evolved from a common ancestor. The four operons may play different roles in the infection process, may be expressed at different times along growth phases or the infection process, or they may simply serve to protect essential virulence genes against mutations. In the present study, the regulation of the expression of the *mce3* operon was investigated, to gain an insight into the role of this operon in *M. tuberculosis*. Our results show the presence of a repressor that controls *mce3* transcription.

METHODS

Bacterial strains and culture media. All cloning steps were performed in *Escherichia coli* DH5 α . *E. coli* BL21(λ DE3) was used for recombinant protein production. Regulation studies

were performed in *Mycobacterium smegmatis* mc²155 or in *M. tuberculosis* H37Rv. *E. coli* was grown in Luria-Bertani (LB) broth or on LB agar. Both mycobacterial species were grown in either Middlebrook 7H9 medium supplemented with 0.05% Tween 80 or Middlebrook 7H11 medium supplemented with oleic acid/albumin/dextrose/catalase (OADC). When necessary, 20 μ g kanamycin ml⁻¹ was added to the media. Electrocompetent *Mycobacterium* cells were prepared according to Parish & Stoker (1998). Mycobacterial strains were transformed by electroporation using a Bio-Rad Gene Pulser, as described by Parish & Stoker (1998). Following electroporation, *M. smegmatis* and *M. tuberculosis* were plated onto Middlebrook 7H11 medium supplemented with OADC and kanamycin.

DNA manipulations. Standard methods were used for restriction-endonuclease digestion of plasmids, for DNA ligations and for other manipulations. Isolation of plasmid DNA was performed using the Wizard Minipreps SV Kit, according to the manufacturer's instructions (Promega). DNA from *M. tuberculosis* was prepared according to van Soolingen *et al.* (1991).

Construction of *lacZ* reporter fusions. Details for the plasmids, vectors and primers used in this study can be found in Table 1. To create fusions with the *E. coli lacZ* gene, the regions containing the *Rv1963*–*Rv1964* intergenic fragment including or not including the *Rv1963* gene were amplified by PCR and cloned into the promoter-probe vector pJEM15 (Timm *et al.*, 1994). The resulting plasmids were called p1963-P3 (plus *Rv1963*) and pP3 (minus *Rv1963*). All amplified fragments were cloned into either pGEM-T (Promega) or pPCRII-TOPO (Invitrogen), before being transferred to pJEM15. Fragments (100 and 206 bp in length) from upstream of the *Rv1964* gene start codon were also fused to a promoterless *lacZ* gene, resulting in plasmids pP3-100 and pP3-200, respectively (Table 1).

The template for PCR was a bacterial artificial chromosome (BAC) containing the *mce3* operon, which was kindly provided by Dr S. T. Cole (Institut Pasteur, France). *M. tuberculosis* genomic DNA was used as a template for amplification of the *mce2* operon.

Different deletions of the *Rv1963*–*Rv1964* intergenic region were obtained as follows. The pPCRII-TOPO intermediate constructs p1963-P3 and pP3 were separately digested with *NheI* and *SalI*; the digest products were then ligated. The inserts from the resulting plasmids were transferred to *Bam*HI-linearized pJEM15 (see Table 1), resulting in p1963-P3 Δ *Nhe* and pP3 Δ *Nhe* (for *NheI* deletions), and p1963-P3 Δ *Sal* and pP3 Δ *Sal* (for *SalI* deletions). An *XmaI*–*AgeI* deletion was obtained by digestion of p1963-P3 with *XmaI* and *AgeI*. The digest products were ligated and introduced into linearized pJEM15, resulting in pP3 Δ *Xma/Age* (Table 1).

Cloning and expression of *Rv1963*. *Rv1963* was amplified from *M. tuberculosis* BAC DNA and cloned into pRSET-A (Table 1). The resulting plasmid, pRSET1963, was introduced into *E. coli* BL21(λ DE3). *E. coli* BL21(pRSET1963) was then grown in LB broth at 28 °C. Expression of the *Rv1963* gene was induced by the addition of 0.1 mM IPTG to the growth medium at the mid-exponential phase of growth. Soluble cell extracts from the culture were prepared by Fast Prep FP120 (Qbiogene) bead-beater disruption (40 s at 6.0 m s⁻¹, using Lysing Matrix B). Proteins separated by SDS-PAGE (Cataldi *et al.*, 1994) were assayed by Western blotting using anti-histidine as the primary antibody (1:3000 dilution; Amersham Pharmacia) and alkaline-phosphatase-conjugated anti-mouse-IgG as the secondary antibody (1:2000 dilution; Sigma).

Table 1. Plasmids, vectors and primers used in this study

Plasmid	Construction and characteristics	Origin		
pJEM15	<i>E. coli</i> –mycobacterial promoter-probe shuttle vector for <i>lacZ</i> fusions; Km ^r	Timm <i>et al.</i> (1994)		
p1963-P3	PCR product from amplification with primers 1963P3up and P3rev was cloned into pPCRII-TOPO and then moved to pJEM15; p1963-P3 carries the <i>mce3</i> promoter region and <i>Rv1963</i>	This study		
pP3	PCR product from amplification with primers P3up and P3rev was cloned into pPCRII-TOPO and then moved to pJEM15; pP3 carries only the <i>mce3</i> promoter region	This study		
p1963-P3Δ <i>Sal</i>	pPCRII-TOPO intermediate of p1963-P3 was digested with <i>Sall</i> , ligated and the <i>Bam</i> HI insert moved to pJEM15; p1963-P3Δ <i>Sal</i> carries an <i>Sall</i> – <i>Sall</i> deletion in the intergenic region and <i>Rv1963</i>	This study		
pP3Δ <i>Sal</i>	pPCRII-TOPO intermediate of pP3 was digested with <i>Sall</i> , ligated and the <i>Bam</i> HI insert was moved to pJEM15; pP3Δ <i>Sal</i> carries an <i>Sall</i> – <i>Sall</i> deletion in the intergenic region	This study		
p1963-P3Δ <i>Nhe</i>	pPCRII-TOPO intermediate of p1963-P3 was digested with <i>Nhe</i> I, ligated and the <i>Bam</i> HI insert was moved to pJEM15; p1963-P3Δ <i>Nhe</i> carries an <i>Nhe</i> I– <i>Nhe</i> I deletion in the intergenic region and <i>Rv1963</i>	This study		
pP3Δ <i>Nhe</i>	pP3 was digested with <i>Nhe</i> I, ligated and the <i>Bam</i> HI insert was moved to pJEM15; pP3Δ <i>Nhe</i> carries an <i>Nhe</i> I– <i>Nhe</i> I deletion in the intergenic region	This study		
pP3Δ <i>Xma</i> /Age	p1963-P3 was digested with <i>Xma</i> I and <i>Age</i> I, ligated and the <i>Bam</i> HI insert was moved to pJEM15; pP3Δ <i>Xma</i> /Age carries an <i>Xma</i> I– <i>Age</i> I deletion, comprising the intergenic region and <i>Rv1963</i>	This study		
pP3-200	PCR product from amplification with primers P3200up and P3revk was cloned into pGEM-T and then moved to pJEM15; pP3-200 carries a 206 bp fragment from upstream of the <i>Rv1964</i> start codon (ATG)	This study		
pP3-100	PCR product from amplification with primers P3100up and P3revk was cloned into pGEM-T and then moved to pJEM15; pP3-100 carries a 100 bp fragment from upstream of the <i>Rv1964</i> start codon (ATG)	This study		
pRSET1963	PCR product from amplification with upregclo and lowregclo inserted into pGEM-T and then moved to the PRSET-A expression vector	This study		
pRSET-A	Expression vector for histidine-tagged proteins; Ap ^r	Invitrogen		
pGEM-T	T/A cloning vector for PCR products; Ap ^r	Promega		
pPCRII-TOPO	T/A cloning vector for PCR products; Ap ^r , Km ^r	Invitrogen		
Primer	Sequence	Restriction site	Annealing temperature (°C)	Amplicon size (bp)
P3up	ggatccCGGCTCGAACCCCCTTCTTGCGCAC	<i>Bam</i> HI	66	896
P3rev	ggatccGGCGCGGCGCACCAGCTGGATTCTGA	<i>Bam</i> HI		
1963P3up	ggatccGGACACCTCATTACACCGATAATG	<i>Bam</i> HI	58	2165
P3rev	ggatccGGCGCGGCGCACCAGCTGGATTCTGA	<i>Bam</i> HI		
P2up	ggtaccTGTTCTAACGATCTTCCGAA	<i>Kpn</i> I	48	139
P2low	ggatccTGCGCCCAGTGTGAATGAAG	<i>Bam</i> HI		
P3100up	ggatccCGTACGCTCACCAATGTCACC	<i>Bam</i> HI	64	100
P3revk	ggtaccGGCGCGGCGCACCAGCTGGATTCTGA	<i>Kpn</i> I		
P3200up	ggatccTACTAGCAAGATACATCATAG	<i>Bam</i> HI	56	206
P3revk	ggtaccGGCGCGGCGCACCAGCTGGATTCTGA	<i>Kpn</i> I		
upregclo	ggatccATGGCATCCGTCGCCCAACCCG	<i>Bam</i> HI	61	1318
lowregclo	gaattcCTATGCGCGCAAGAGGTTACCT	<i>Eco</i> RI		
Low861	GGATTTCGATGCTGGGCCGTT	NA	56	na

NA, Not applicable, as used for primer-extension experiments.

PCR amplification. PCR amplifications were performed using *Taq* DNA polymerase (Promega) under standard conditions in a total volume of 50 µl. dNTPs were used at a concentration

of 0.2 mM each; 20 pmol of each primer was used. The protocol used for amplification was as follows; denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at

94 °C for 1 min, 1 min annealing at a temperature dependent on the primer pair used (Table 1) and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 10 min. A total of 2 ng of genomic *M. tuberculosis* DNA or BAC DNA was used as template.

RNA preparation. Total RNA from *M. tuberculosis* H37Rv was isolated using the FastRNA Kit-Blue (Qbiogene). Briefly, 50 ml of a culture was harvested during the exponential phase of growth by centrifugation at 3000 r.p.m. for 10 min. The cell pellet was resuspended in 100 µl diethyl pyrocarbonate (DEPC)-treated water and transferred to a 2 ml screw-cap microcentrifuge tube containing 0.1 mm diameter zirconium beads and FastRNA reagents (Qbiogene). Cells were disrupted by using a Fastprep FP120 bead-beater for 20 s at a speed of 6.0 m s⁻¹. Total RNA was then extracted from the cells following the manufacturer's instructions. After agarose-gel electrophoresis of the total RNA and staining of the gels with ethidium bromide, the different bands of rRNA were clearly visible, indicating that the RNA preparations were of a high integrity.

Primer extension. This was performed using the primer Low861 (Table 1). Ten picomoles of the non-phosphorylated primer were labelled by using T4 polynucleotide kinase (Promega) in the presence of [γ -³²P]ATP. The specific activity of the primer was 8000 c.p.m. pmol⁻¹. *M. tuberculosis* H37Rv RNA (6 µg) and the labelled primer (0.1 pmol, 33000 c.p.m. pmol⁻¹) were mixed in 7 µl of 50 mM Tris/HCl (pH 8.3) containing 0.1 M KCl. The reaction was then incubated at 94 °C for 1 min, at 56 °C for 10 min and then on ice for 15 min. The mixture was adjusted to a final volume of 12 µl by the addition of 1 µl of a mixture containing the dNTPs (2.5 mM each), 0.5 µl of RNasin (Promega), 2.2 µl of 5× reverse transcriptase buffer [0.25 M Tris/HCl (pH 8.3), 0.2 M KCl, 36 mM magnesium acetate, 0.01 M DTT], 0.8 µl of DEPC-treated water and 0.5 µl avian myeloblastosis virus reverse transcriptase (Promega). Reverse transcription was performed at 42 °C for 45 min; the reaction was stopped by the addition of 5 µl of stop buffer to the reaction mixture.

The reverse-transcription products were separated by PAGE (6% acrylamide gel containing 8 M urea), and were run alongside the sequencing products obtained with the Low861 primer. The gels were fixed by immersion in a mixture containing 5% (v/v) methanol and 5% (v/v) acetic acid. They were then exposed to X-ray film (Kodak X-Omat RS) for 24 h at -70 °C.

Computer analyses. Amino-acid-sequence alignments were generated by searching public databases using BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST>). *M. smegmatis* BLAST searches (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>) were used to identify an *Rv1963* orthologue in this bacterium. Domain analysis was performed by using PFAM (<http://www.cgr.ki.se/Pfam>). Prokaryotic promoter sequences were searched by using the prokaryotic option of the Neural Network Promoter Prediction algorithm of the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/seq_tools/promoter.html).

Measurements of β -galactosidase activity. For *in vitro* determinations of β -galactosidase activity, 1 ml of a recombinant *M. smegmatis* culture or 1 ml of a recombinant *M. tuberculosis* culture was pelleted, resuspended in 1 ml buffer Z [0.1 M sodium phosphate (pH 7.0), 10 mM KCl, 1 mM MgSO₄ and 50 mM mercaptoethanol], disrupted using a Fast Prep FP120 bead-beater (40 s at 6.0 m s⁻¹, using Lysing Matrix B) and centrifuged at 13000 r.p.m. to obtain the soluble

cell extract. β -Galactosidase measurements were performed on the soluble cell extract, as described by Miller (1972); results are expressed in Miller units [$A_{420} \times 1000/\text{reaction time (min)} \times A_{550}$].

For determination of the β -galactosidase activity in an infected macrophage cell line, the murine macrophage-like cell line J774 was cultivated in RPMI medium 1640 with 25 mM HEPES buffer and L-glutamine supplemented with 10% fetal calf serum in 5% CO₂ at 37 °C in T25 flat-bottomed cell-culture flasks. The cell line was incubated for 18–24 h until a density of 5×10^6 cells per flask was reached. Recombinant *M. tuberculosis* H37Rv strains were grown in Middlebrook 7H10 medium containing 20 µg kanamycin ml⁻¹. The cultures were then harvested, resuspended in RPMI medium, vortexed, sonicated for 1 min in an ultrasonic cleaner and allowed to settle. The upper part of each of the bacterial suspensions (supposedly free of clumps of bacteria) was used to infect the J774 cell line. The OD₆₅₀ value for the upper portion of the suspension was taken, and it was assumed that an OD₆₅₀ value of 0.1 was equal to 10⁷ c.f.u. ml⁻¹ (data not shown). This suspension was then used to replace the medium that the J774 cells were in, and infection of the J774 cells was performed at an m.o.i. of 100. Infected cells were incubated for 3 h, then washed five times with fresh RPMI medium. At 0, 1, 4 and 6 days post-infection, the J774 cells were scraped and lysed with 1 ml of 1% Triton X-100 for 15 min. The resulting suspension was centrifuged to obtain the mycobacterial pellet, and β -galactosidase activity was determined for the mycobacterial cells, as described above. Activity was related to the number of bacteria as determined by plating onto Middlebrook 7H11 agar supplemented with kanamycin and OADC. As a control, the β -galactosidase activity of the bacterial strains that were used for macrophage infection but grown *in vitro* was determined.

Gel-shift assay. A 100 bp and a 206 bp fragment from upstream of the start codon of *Rv1964* (from *mce3*; see 'Construction of *lacZ* reporter fusions') and a 139 bp fragment from upstream of *Rv0586* of the *mce2* promoter region (obtained using primers P2up and P2low; Table 1) were used as probes in this assay. Fragments were labelled with [γ -³²P]ATP by using the polynucleotide kinase enzyme. Non-incorporated nucleotides were eliminated from the mixture containing the labelled probes by using Wizard PCR Prep Columns (Promega). Labelled probes were incubated with the soluble cell extract from recombinant *E. coli* BL21 over-expressing *Rv1963* or from *E. coli* BL21(pRSET) (negative control) in binding buffer [4% glycerol (v/v), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris/HCl (pH 7.5), 0.05 mg salmon sperm DNA ml⁻¹] for 20 min at room temperature. The samples were separated by PAGE [4% acrylamide gel containing 1× TBE (0.05 M Tris base, 0.05 M boric acid, 1 mM EDTA-Na₂·2H₂O), 0.1% bisacrylamide and 2.5% glycerol] for 3 h at 100 V, after a 30 min pre-run in a 0.5× TBE buffer tank. The gels were then dried and exposed to X-ray film.

RESULTS

Characterization of a putative regulatory gene for the *mce3* operon

The *Rv1963* gene is located upstream of the *mce3* operon (Fig. 1) and encodes a hypothetical protein of 44.3 kDa (407aa). This ORF is homologous to the TetR family of regulators from several bacteria. The best

Fig. 1. Schematic representation of the *Rv1963*–*Rv1964* intergenic region. Thick, solid lines represent the genes; the arrows indicate the direction of transcription. The open box represents the promoter-containing region. The primers used in amplifications, and their direction of action, are shown below the schematic. The –100 and –206 positions upstream of the *Rv1964* start codon (ATG) are shown. *Rv1964* is the first ORF of the *mce3* operon. The restriction sites used for subcloning of the promoter region are also shown.

Fig. 2. Effect of *Rv1963* on *mce3* promoter activity. Comparison of *mce3* promoter activity during the growth of recombinant (a) *M. smegmatis* and (b) *M. tuberculosis* transformed with pJEM15 (vector alone as control), pP3 (construct without *Rv1963*) or p1963-P3 (construct with *Rv1963*). In both (a) and (b) the bars represent β -galactosidase activity; solid bars represent p1963-P3 and grey bars represent pP3. pJEM15 is represented by open bars in (a) and by hatched bars in (b). Growth curves for the recombinant strains are also shown in (a) and (b); $\blacktriangle$, strain transformed with pJEM15; $\blacksquare$, strain transformed with p1963-P3; $\bullet$, strain transformed with pP3.

BLAST hits in GenBank were with putative regulatory proteins from *Streptomyces coelicolor* (accession no. AL163641; 42% similarity, E-value 6e-24) and *M.*

tuberculosis (accession no. AE007094; 43% similarity, E-value 1e-08). These data suggest that the *Rv1963*-encoded protein could regulate *mce3* transcription. Sequence analysis of the *Rv1963* gene by PFAM showed two *tetR*-family signatures, one located at amino acids 19–65 and one, with a lower matching, located at amino acids 223–269. A helix–turn–helix motif was identified at amino acids 36–57.

The *Rv1963* protein downregulates *mce3* transcription

To study the role of the putative regulatory gene *Rv1963* in *mce3* transcription, mycobacteria harbouring gene fusions between the *mce3* promoter region and the *Escherichia coli lacZ* gene, either containing or not containing the *Rv1963* gene, were used. *M. smegmatis* mc²155 was transformed with these constructs and the β -galactosidase activity of the *M. smegmatis* transformants was measured throughout the growth of the cultures. β -Galactosidase activity was much higher in *M. smegmatis* mc²155(pP3) (minus *Rv1963*) than in *M. smegmatis* mc²155(p1963-P3) (plus *Rv1963*) (Fig. 2a). Significantly, a similar difference in activity was observed in *M. tuberculosis* H37Rv transformed with the same constructs (Fig. 2b). These results indicate that the *Rv1963*-encoded protein negatively regulates the transcription of the *mce3* operon. Consequently, we propose to redesign *Rv1963* as Mce3R. β -Galactosidase activity also greatly increased during the stationary phase of growth for *M. smegmatis* mc²155(p3) (Fig. 2a). A similar increase in β -galactosidase activity was also observed for *M. smegmatis* mc²155(p1963-P3), although this activity was still at a lower level than seen for *M. smegmatis* mc²155(p3). The difference between the β -galactosidase activities of *M. smegmatis* mc²155(p3) and *M. smegmatis* mc²155(p1963-P3) remained approximately the same (17-fold) along the growth curves of the two strains. In the *M. tuberculosis* transformants, an increase in *mce3* expression was also observed during the stationary phase, but was followed by a decline during the later periods of this growth phase (Fig. 2b).

Mapping of the *mce3* promoter

To map the *mce3* promoter, different segments of the *mce3R*–*Rv1964* intergenic region were assayed for their promoter activity (Fig. 3). A strain harbouring pP3 Δ Xma/Age expressed high β -galactosidase activity (1100 Miller units). This result could be explained by the elimination of the *mce3R* repressor and suggests that the *mce3* promoter is located between the AgeI site and the *Rv1964* start codon. Deletion of a SalI–SalI fragment (Δ Sal) from the intergenic region abolished the expression of *mce3*, even in a strain lacking the repressor gene, suggesting that the promoter is located between the SalI sites or that it spans one of the SalI sites. Finally, strains harbouring plasmids with a NheI deletion (Δ NheI) showed no alteration in their β -galactosidase activities, demonstrating that the NheI–NheI segment

Fig. 3. Promoter activity of different segments of the *Rv1963-Rv1964* intergenic region. Restriction sites used for deletion of segments are marked on the intact construct (p1963-P3). Deletions are marked by dotted lines; *lacZ* is represented by an open arrow; *Rv1963* is represented by a solid arrow.

does not contain the *mce3* promoter nor the repressor-binding region. To further localize the *mce3* promoter of *M. tuberculosis*, 206 and 100 bp fragments from upstream of the start codon (ATG) of *Rv1964* were evaluated for their promoter activity. To do this, these fragments were cloned into pJEM15 (generating pP3-200 and pP3-100, respectively) and tested in *M. smegmatis* mc²155. β-Galactosidase activity was 80-fold higher in *M. smegmatis* mc²155(pP3-200) than in *M. smegmatis* mc²155(pP3-100), indicating that a functional *mce3* promoter is located between 100 and 206 bp upstream of the *Rv1964* start codon.

Primer-extension experiments (Fig. 4a) indicated that the transcription start point (TSP) of the *mce3* promoter was a C base 143 nt upstream of the *Rv1964* start codon. A neural-network on-line program that searches for putative prokaryotic promoters located a probable promoter extending from -170 to -230 upstream of the ATG start codon corresponding to the first *mce3* ORF (*Rv1964*). DNA STRIDER software identified putative -35 and -10 sequences in the proposed *mce3* promoter region (Fig. 4b), a probable -35 sequence, TAGCAA (extending from -204 to -198), with 4/6 matches with the -35 consensus sequences of the *hsp60* promoter of BGC (TTGCAC) (25; 27), and a probable -10 sequence, TATATG (extending from -182 to -178), revealing 6/6 matches with the typical *E. coli* -10 consensus sequence. The localization of these putative promoter hexamers coincides with the putative promoter found by the neural network. However, the -10 element is located at -32 bp of the TSP. One less consensual sequence, TATTTA, is localized 12 bp upstream of the proposed TSP. A putative RBS was found by visual analysis of the downstream region of the proposed TSP (Fig. 4b).

Expression of the *mce3* operon in the cultured J774 cell line

To determine whether expression of the *mce3* operon was intracellularly induced, *M. tuberculosis* H37Rv transformed with p1963-P3, pP3 or pJEM15 was used to infect the J774 macrophage-like cell line. The bacterial cells used for infection came from cultures at an OD₆₅₀ value of 1.2 for *M. tuberculosis* H37Rv(pJEM15), 1.4 for *M. tuberculosis* H37Rv(pP3) and 1.7 for *M. tuberculosis* H37Rv(p1963-P3). At 0, 1, 4 and 6 days post-infection, cell lysates of the three transformants were prepared and their β-galactosidase activities were measured. The highest β-galactosidase activity was observed during the late stages of infection (4 and 6 days post-infection) in *M. tuberculosis* H37Rv(pP3) and *M. tuberculosis* H37Rv(p1963-P3). Induction of the *mce3* operon in the infected J774 cell line was suggested by the comparison of β-galactosidase activity *in vitro* (bacteria used for infection) to that at 6 days post-infection. However, the *M. tuberculosis* H37Rv(pP3) to *M. tuberculosis* H37Rv(p1963-P3) ratio of β-galactosidase activity was similar in bacteria growing intracellularly and in those growing *in vitro* (around 8:1, Table 2), indicating that intracellular growth does not induce removal of repression in the experimental conditions used here.

Binding of the Mce3R protein to the *mce3* promoter region

The Mce3R protein was examined for its ability to bind to the *mce3* promoter region by performing a gel-shift assay. *mce3R* was cloned into the expression vector pRSET-A; high expression recombinant *mce3R* was then induced in *E. coli* BL21(pRSET1963) by the addition of IPTG to the growth medium (Fig. 5). Since

(b)

TACTAGCAAAGATACATCATAGCCAATATATGCCAGTTTGCA
 TTGCTATTTACCGATCAGTTGTGCAAGCAATCGCGTATTGG
 CTATGGACATCAGCGGTTCTGCCGCGTACGCTACCAATG
 TCACCGATCGTCGACCTGTCCGGGGGGCCAGCGTGCGCC
 ACCTCACCCAACGGCCAGCATCGAATCCAGCTGGTGCGC
 CGCGCC

Fig. 4. (a) TSP as determined by primer extension. The TSP is indicated by an arrow; sequencing products generated by using the Low861 primer are shown alongside. (b) Predicted promoter region of the *mce3* operon. The -10 (TATATG) and -35 (TAGCAA) regions are highlighted; an alternative -10 element (TATTTA) is also highlighted. A double-underlined C shows the TSP, as determined by primer extension. Letters in italics indicate putative ribosome-binding sites.

the formation of inclusion bodies by the bacterium prevented the purification of histidine-tagged Mce3R on nickel/agarose columns, we decided to work with the soluble fraction of the cell extract of *E. coli* BL21(pRSET1963), which contained Mce3R (Fig. 5). Three different probes were tested in gel-shift assays on

Fig. 5. Overexpression of Mce3R in *E. coli* BL21. Expression was induced by the addition of IPTG to the growth medium; Western blotting was done using an anti-His mAb. Lanes: 1, molecular mass markers; 2, soluble fraction of cell extract of *E. coli* BL21(pRSET1963); 3, whole-cell extract of *E. coli* BL21(pRSET-A); 4, insoluble cell extract of *E. coli* BL21(pRSET1963); 5, Coomassie-blue-stained cell extract of *E. coli* BL21(pRSET1963).

the cell extract of *E. coli* BL21(pRSET1963); these were 100 and 206 bp fragments upstream of the start codon (ATG) of *Rv1964* and a 139 bp fragment from the *mce2* promoter region. The 206 bp fragment showed Mce3R binding to the *mce3* promoter region in a dose-dependent manner (Fig. 6), whereas no binding was observed with the 100 bp fragment. A cell extract from *E. coli* BL21 transformed with vector alone produced no retardation on the 206 bp fragment. Interestingly, Mce3R bound to the 139 bp *mce2* fragment, suggesting a regulatory action of Mce3R on the *mce2* promoter; however, the strength of this binding was lower than seen for Mce3R and the *mce3* promoter.

DISCUSSION

Currently, the physiological function of Mce proteins is not known, although a protein promoting mammalian cell entry, Mcep (Arruda *et al.*, 1993), is encoded by the *mce1* operon of *M. tuberculosis*. Mce proteins seem to be exported by the bacterium, as suggested by their

Table 2. Effect of *Rv1963* expression on *mce3* promoter activity in recombinant strains used to infect J774 cell line

J774 Macrophage-like cell line infected with	<i>In vitro</i> β -galactosidase activity (Miller units)*	β -Galactosidase activity (Miller units) at			
		0 d.p.i.†	1 d.p.i.	4 d.p.i.	6 d.p.i.
<i>M. tuberculosis</i> H37Rv(pJEM15)	2	22	20	185	142
<i>M. tuberculosis</i> H37Rv(p1963-P3)	21	49	55	454	1987
<i>M. tuberculosis</i> H37Rv(pP3)	140	541	588	4684	14111

* Activity of bacterial cultures used as inoculum.

† d.p.i., Days post-infection.

Fig. 6. DNA binding activity of Mce3R. Gel-shift analysis of different *mce3* and *mce2* promoter-containing regions. In (a), a 100 bp fragment amplified from the *mce3* promoter region was used as the probe. Lanes: 1, probe incubated with PBS; 2, probe incubated with 16 µg of soluble cell extract from *E. coli* BL21(pRSET1963); 3, probe incubated with 32 µg of soluble cell extract from *E. coli* BL21(pRSET1963). In (b), a 206 bp fragment amplified from the *mce3* promoter region was used as the probe. Lanes: 4, probe incubated with PBS; 5, probe incubated with 27 µg of soluble cell extract from *E. coli* BL21(pRSET1963); 6, probe incubated with 54 µg of soluble cell extract from *E. coli* BL21(pRSET1963); 7, probe incubated with 108 µg of soluble cell extract from *E. coli* BL21(pRSET1963); 8, probe incubated with 30 µg of soluble cell extract from *E. coli* BL21(pRSET-A). In (c), a 139 bp fragment amplified from the *mce2* promoter region was used. Lanes: 9, probe incubated with PBS; 10, probe incubated with 27 µg of soluble extract from *E. coli* BL21(pRSET1963); 11, probe incubated with 54 µg of soluble extract from *E. coli* BL21(pRSET1963); 12, probe incubated with 108 µg of soluble extract from *E. coli* BL21(pRSET1963); 13, probe incubated with 30 µg of soluble cell extract from *E. coli* BL21(pRSET-A). Details for the probes used can be found in Methods.

predicted topology and by subcellular fractionation experiments (data not shown). The membrane localization of Mce proteins predicts a function for them in transmembrane transport, cell-wall synthesis or other functions associated with the bacterium–environment relationship. Genes encoding proteins homologous to those encoded by the *mce* operons are also present in other mycobacterial species, such as *M. avium* (Parker *et al.*, 1995), *M. smegmatis* (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>) and *M. leprae* (Cole *et al.*, 2001). However, *mce3* is absent from *M. bovis* and *M. microti*. The biological consequence of the absence of *mce3* in these species is unclear, but it is important to note that although the *mce3* operon is deleted in *M. bovis*, *Rv1963* is conserved within this species. In place of the *mce3* operon ORFs, the *M. bovis* genome has a chimeral ORF between *Rv1964* and *Rv1977*. *M. smegmatis* also contains four operons with a structure similar to the *mce* operons of *M. tuberculosis* (data not shown). Surprisingly, in the *M. smegmatis* genome the gene most similar to *Rv1963* is not upstream of the *mce3*-like operon, but is instead elsewhere in the genome. Also, upstream of the *mce3*-like operon there is another *tetR*-like regulator with a lesser homology to *Rv1963* (data not shown).

Even though the expression of Mce proteins has been demonstrated both *in vitro* (Harboe *et al.*, 1999) and *in vivo* (Ahmad *et al.*, 1999), nothing is known about the regulation of production of Mce proteins. The observation of a regulatory-type gene in the vicinity of the *mce3* operon prompted us to determine whether this putative regulator controls *mce* expression. The 0.9 kbp intergenic region between *Rv1963* and *Rv1964* should be the location of the *mce3* promoter and the target of regulatory proteins. Progressive deletions and cloning of this region helped us to map the promoter region in a fragment extending from the *Rv1964* start codon to 206 bp upstream of it. A neural-network search for putative prokaryotic promoters located a probable promoter extending from –170 to –230 upstream of the ATG start of the first *mce3* ORF. As deduced from gel-shift assays, it was also observed that Mce3R binds to this region.

Using all of the published putative mycobacterial sequences, Mulder *et al.* (1997) calculated the mycobacterial promoter to consist of a –35 consensus sequence, TTGACG/A, and a –10 consensus sequence, TATA/GA/CT. Putative –10 (TATATG) and –35 (TAGCAA) sequences were identified in the predicted promoter region of the *mce3* operon. The proposed –10 hexamer is identical to the corresponding sequence in *E. coli* and conserves the four first positions of the Mulder *et al.* (1997) consensus sequence. The putative –35 sequence conserves three positions of the Mulder *et al.* (1997) consensus sequence and is identical to other individual mycobacterial promoters. However, the proposed –10 sequence is far (32 bp) from the TSP identified by primer-extension experiments. Possible explanations for this large separation could be (i) an erroneous TSP was mapped because the 5' end of the transcript was processed or degraded, or (ii) non-conserved –10 and –35 sequences are present in the *mce3* promoter region. As in many actinomycete promoters, mycobacterial promoters not carrying the canonical –35 and –10 sequences have been reported. Counting upstream from the proposed start site of the *mce3* promoter region there is another, less consensual, sequence, TATTTA, at a distance of –12 bp.

In general, the TetR family of bacterial regulators are repressors; many of these proteins regulate genes that encode proteins involved in membrane processes such as osmoregulation (Rkenes *et al.*, 1996), permeability (Ma *et al.*, 1996; Lucas *et al.*, 1997; Namwat *et al.*, 2001) and resistance to antibiotics or quaternary ammonium salts (Rouch *et al.*, 1990; Grkovic *et al.*, 1998), among other functions. The prototype TetR repressor is TetR from the Tn10 transposon of *E. coli* (Orth *et al.*, 2000). There are approximately 40 *tetR*-family regulatory genes in *M. tuberculosis* (http://www.sanger.ac.uk/Projects/M_tuberculosis). To the best of our knowledge, the *Rv1963*-encoded protein examined in this study (as Mce3R) is the first TetR-like regulatory protein whose function has been described in *M. tuberculosis*. As with other TetR regulators, a helix–turn–helix motif and a *tetR*-family signature sequence reside in the first third of

the *Rv1963* gene. Most TetR-like regulators have molecular masses ranging from 21 to 25 kDa; however, Mce3R (*Rv1963*) has a molecular mass >25 kDa. We observed that other TetR-like proteins with molecular masses >25 kDa contain two consensus signatures, as does Mce3R (*Rv1963*). The last third of Mce3R (*Rv1963*) shows no homology to other proteins. The two TetR-like regulators most homologous to Mce3R (*Rv1963*), *Rv2506* and *Rv3557c*, are in the vicinity of lipid-metabolism-related genes of the *M. tuberculosis* genome.

We could not find the inducer(s) of the *mce3* operon; different conditions of stress (ethanol, SDS or low carbon) did not abolish repression of expression (data not shown). No removal of repression was observed when the J774 macrophage-like cell line was infected with recombinant *M. tuberculosis*. The possibility exists that removal of repression could not be observed due to the fact that in recombinant mycobacteria carrying the reporter gene the repressor is harboured *in cis* in multicopy plasmids. However, an increase in *mce3* expression by *M. tuberculosis* H37Rv(pP3) and *M. tuberculosis* H37Rv(p1963-P3) was observed in the later stages of J774 infection, suggesting that additional factors may affect *mce3* expression.

At present, it is not known whether the *Rv1963*-encoded protein regulates genes other than *mce3*, but gel-shift experiments indicated that Mce3R binds to the *mce2* promoter region, raising the possibility that *mce2* may also be negatively regulated by the *Rv1963*-encoded protein. However, the binding of the recombinant protein Mce3R to the *mce2* promoter seems to be weaker than binding of this protein to the *mce3* promoter, suggesting that fine-tuning of regulation of the *mce* operons may occur. Interestingly, a moderately homologous region of about 100 bp was found in the promoter-containing regions of *mce2* and *mce3* (data not shown).

The present study reveals interesting features of the control of expression of the *mce* operons of *M. tuberculosis*. It also poses a number of questions that need to be answered, such as (i) does the *Rv1963*-encoded regulatory protein have the same effect on *mce* promoters other than *mce3*, and (ii) which extracellular signals are required for *mce* expression? At present, it is not known whether the *mce1* and *mce4* promoter regions are also the target of the *Rv1963*-encoded trans-acting regulatory factor, but the high homology between the *mce* operons supports the idea of a broader regulatory mechanism.

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