Regulation of the expression of *whiB1* in *Mycobacterium tuberculosis*: role of cAMP receptor protein

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The *wbl* (*whiB*-like) genes encode putative transcription factors unique to actinomycetes. This study characterized the promoter element of one of the seven *wbl* genes of *Mycobacterium tuberculosis*, *whiB1* (Rv3219c). The results reveal that *whiB1* is transcribed by a class I-type cAMP receptor protein (CRP)-dependent promoter, harbouring a CRP-binding site positioned at –58.5 with respect to its transcription start point. *In vivo* promoter activity analysis and electrophoretic mobility shift assays suggest that the expression of *whiB1* is indeed regulated by cAMP-dependent binding of CRP^M (encoded by the *M. tuberculosis* gene Rv3676) to the *whiB1* 5' untranslated region (5'UTR). β -Galactosidase gene fusion analysis revealed induction of the *whiB1* promoter in *M. tuberculosis* on addition of exogenous dibutyric cAMP (a diffusible cAMP analogue) only when an intact CRP-binding site was present. These results indicate that *M. tuberculosis whiB1* transcription is regulated in part by cAMP levels via direct binding of cAMP-activated CRP^M to a consensus CRP-binding site in the *whiB1* 5'UTR.

INTRODUCTION

Mycobacterium tuberculosis, the aetiological agent of tuberculosis, accounts for nearly 2 million human deaths every year. Approximately one-third of the world's population is latently infected with *M. tuberculosis*, and 7 to 8 million new TB cases occur annually (Dye *et al.*, 1999). *M. tuberculosis* can survive in diverse surroundings ranging from the human host to droplet nuclei in the atmosphere. Adaptation to such diverse conditions requires controlled regulation of the expression of key genes that allow the bacillus to alter its physiology in response to changes in environmental stimuli. Sequencing of the *M. tuberculosis* genome has revealed the presence of more than 100 regulatory proteins, 13 sigma factors and 11 two-component systems (Cole *et al.*, 1998), which provide the bacterium with a high degree of adaptability.

The Wbl (WhiB-like) family of proteins is present throughout the actinomycetes but absent from all other organisms evaluated so far (Molle *et al.*, 2000; Soliveri *et al.*, 2000). Due to the presence of a conserved helix–turn–helix motif, these proteins are believed to function as DNA-binding transcription regulators. The first of these proteins, WhiB, was identified in *Streptomyces coelicolor*, a Gram-positive

sporulating bacterium closely related to *M. tuberculosis*. *S. coelicolor whiB* mutants produce abnormally long, tightly coiled aerial hyphae that are completely blocked in their ability to form sporulation septa (Chater, 1972; Davis & Chater, 1992; Flardh *et al.*, 1999). Studies of *whiB* orthologues in mycobacteria have shown that the *M. smegmatis whiB2* gene (also called *whmD*) is essential (Gomez & Bishai, 2000); *M. tuberculosis whiB3* plays a role in virulence and its gene product may interact with a sigma factor of RNA polymerase (Steyn *et al.*, 2002); *whiB7* of *M. tuberculosis* is involved in multi-drug resistance (Morris *et al.*, 2005). Each of the Wbl family of proteins contains four invariant cysteine residues, which are believed to be involved in binding a [4Fe–4S] cluster (Jakimowicz *et al.*, 2005). The functional importance of this cluster is emphasized by the observation that none of the four *whiD* alleles carrying mutations at these cysteine residues was able to complement the *whiD* mutant phenotype in *S. coelicolor* (Jakimowicz *et al.*, 2005).

It has recently been reported that the *M. tuberculosis* gene Rv3676 encoding a CRP-FNR family protein (hereafter referred to as CRP^M) is defective in the vaccine strain *Mycobacterium bovis* BCG (Spreadbury *et al.*, 2005). Moreover, an *M. tuberculosis* mutant lacking an intact Rv3676 gene is attenuated for virulence and shows reduced expression of several mycobacterial genes including resuscitation promoting factor, *rpfa* (Rickman *et al.*, 2005). One of the genes that was dependent on the presence of Rv3676 for full expression was *whiB1*, suggesting the role of cAMP in the expression of *whiB1* in *M. tuberculosis*.

Abbreviations: CRP, cAMP receptor protein; CRP^M, CRP encoded by the *M. tuberculosis* gene Rv3676; db-cAMP, dibutyric cAMP; EMSA, electrophoretic mobility shift assay; 5'-RACE, rapid amplification of cDNA ends; TSP, transcription start point; 5'UTR, 5' untranslated region.

In this study we show that *whiB1* is transcribed by a class I-type CRP-dependent promoter. We provide evidence that *whiB1* transcription is regulated by varying cAMP levels via a mechanism involving direct binding of cAMP-activated CRP^M to a consensus site adjacent to the *whiB1* promoter.

METHODS

Bacterial strains, plasmids and growth conditions. In this study we used *Escherichia coli* strain DH5 α (F'*lndA1 hsdR17* [*r_k⁻ m_k⁺*] *glnV44 thi-1 recA1 gyrA* [Nal^r] *relA1* Δ [*lacIZYA-argF*]U169 *deoR* [ϕ 80*dlac*(*lacZ*)M15]), from Stratagene, and BL21(DE3) (F⁻ *ompT hsdS_B* [*r_B⁻ m_B⁻*] *gal dcm* [DE3]), from Novagen. *M. tuberculosis* CDC1551 was obtained from Colorado State University, CO, USA. Plasmid pSD5B (mycobacteria-*E. coli* shuttle vector carrying promoterless *lacZ* gene, Km^R; Jain *et al.*, 1997) was kindly provided by Dr Anil Tyagi, University of Delhi South Campus, New Delhi, India, and pET-22a (expression vector with pBR322 origin of DNA replication and T7 promoter, Ap^R) was obtained from Novagen. Luria-Bertani (LB) broth and LB agar media were used for culturing *E. coli*, and 7H9 broth and 7H10 agar, both supplemented with 1 $\times$ OADC were used for culturing mycobacteria. Both *E. coli* and mycobacteria were grown at 37 °C with shaking at 200 r.p.m. For cAMP treatment, the *M. tuberculosis* cells were cultured in 7H9 medium supplemented with 1 $\times$ OADC (oleic acid/albumin/dextrose/catalase) and harvested at the desired OD₆₀₀. The cell pellet was resuspended in 0.1 vol. 0.125 M potassium phosphate buffer, pH 7.4, and divided into two equivalent portions. One of these was incubated with a cell-diffusible form of cAMP, dibutyryl cAMP (db-cAMP) (20 mM) and the other with an equivalent concentration of butyric acid (20 mM). After incubation for 2 h at 37 °C with shaking, the cell pellets were harvested and stored at -70 °C. Antibiotics were added when necessary: ampicillin (50 μ g ml⁻¹) and kanamycin (25 μ g ml⁻¹ for *E. coli* and 15 μ g ml⁻¹ for mycobacteria).

DNA techniques. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and *Taq* polymerase was purchased from Invitrogen. Protocols for DNA manipulations, including plasmid DNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, and isolation and ligation of DNA fragments were as described by Sambrook *et al.* (1989). *E. coli* DH5 α was transformed by the standard protocol (Sambrook *et al.*, 1989) and *M. tuberculosis* by electroporation. PCR amplifications were carried out according to the manufacturer's specifications (Applied Biosystems). Each of the 30 cycles was carried out at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. DNA fragments used for cloning and labelling reactions were purified by using the Qiagen gel extraction kit according to the manufacturer's specifications.

Mapping the 5' end of the *whiB1* mRNA. The 5'-RACE (rapid amplification of cDNA ends) technique (Frohman, 1993) was used to determine the transcription initiation site with a kit purchased from Invitrogen. Total RNA was isolated from *M. tuberculosis* (mid-exponential phase) by using the Trizol method according to the instructions provided by the supplier (Invitrogen). The abridged anchor primer (AAP) and abridged universal amplification primers (AUAP) were used in combination with the gene-specific primers. The gene-specific primers used for RT-PCR, nested PCR1 and nested PCR2 were *whiB1*/RACE1 and *whiB1*/RACE2, respectively (see Fig. 1b). The PCR products were subsequently sequenced by using the gene-specific primer *whiB1*/RACE2.

Construction of P_{*whiB1*}-*lacZ* transcriptional fusion construct and measurement of β -galactosidase activity. A DNA fragment ~250 bp in length containing the *whiB1* promoter sequence from

positions -214 to +33 with respect to the transcriptional start point was PCR-amplified from chromosomal DNA of *M. tuberculosis* using the primers P*whiB1*(F) (carrying an *Xba*I site) and P*whiB1*(R) (carrying an *Sph*I site) (Fig. 1b). A transcriptional fusion construct was generated by cloning the PCR amplicon in pSD5B at the *Xba*I and *Sph*I sites, and the recombinant vector was introduced into *M. tuberculosis* by electroporation. Promoter activity was determined by β -galactosidase assays using cell lysates of the cultures harvested at their exponential phase of growth. Assays were performed as described by Miller (1972); the values presented are means of three separate assays.

Generation of point mutations. DNA fragments carrying the mutations in promoter sequences were constructed by two-step PCR amplification using overlapping modified primers, as described earlier (Good & Nazar, 1992). After cleavage with *Xba*I/*Sph*I, these fragments were cloned in pSD5B, the recombinant vectors were used to transform the *M. tuberculosis* CDC1551 strain, and the transformants were grown on 7H10 agar plates containing kanamycin (25 μ g ml⁻¹) and X-Gal (60 μ g ml⁻¹).

Purification of CRP^M of *M. tuberculosis*. The gene encoding the CRP^M of *M. tuberculosis* was PCR-amplified from *M. tuberculosis* chromosomal DNA using gene-specific primers, Pr3676(F) (5'-GGGGCATATGGACGAGATCCTGGCCAGGG-3') and Pr3676(R) (5'-GGGGCTCGAGCCTCGCTCGGCGGGCCAGTCT-3'). These primers were selected such that the amplicons carried the sequence of the complete ORF excluding the termination codon. The amplicons were cloned into the expression vector pET-22a at the *Nde*I and *Xho*I sites. The resulting construct pET*crp* was used to transform *E. coli* BL21(DE3). CRP with histidine tags at the C-terminus was purified from the transformants by Ni-NTA chromatography according to the manufacturer's protocol (Qiagen). Purity of the protein was analysed by SDS-PAGE.

Electrophoretic mobility shift assay. DNA probes used for electrophoretic mobility shift assay (EMSA) analysis were prepared by PCR amplification of the desired regions of the *whiB1* promoter, using 5'-end biotin-labelled synthetic oligonucleotides as the primers. The amplicons were purified from agarose gels and used for gel-shift experiments. The EMSA reaction mixture (10 μ l) contained 20 fmol biotin-labelled probe and CRP^M in binding buffer (40 mM HEPES/NaOH pH 7.5, 50 mM KCl, 2 mM MgCl₂, 0.01 mM EDTA, 1 mM DTT and 1 mg BSA ml⁻¹) containing 1 μ g nonspecific competitor DNA poly(dI-dC) (Amersham Pharmacia Biotech) and 1 mM cAMP. Following incubation at 37 °C for 15 min, the DNA-protein complexes were resolved in a native 5% polyacrylamide gel (acrylamide:bisacrylamide, 30:1, w/w) in 0.5 $\times$ TBE buffer (45 mM Tris base, 45 mM boric acid and 1 mM EDTA, pH 8.0). Samples were electrophoresed at 100 V, and transferred to positively charged nylon membranes (Amersham Pharmacia Biotech). After UV cross-linking (100 μ J cm⁻² for 2 min), the biotinylated probes in the membrane were detected using the LightShift Chemiluminescent EMSA kit (Pierce). Each EMSA was performed three times to confirm CRP^M-DNA binding.

RESULTS

The upstream region of *whiB1* possesses a CRP-binding site

Sequence analysis of the upstream region of the *M. tuberculosis whiB1* gene indicated the presence of a putative CRP-binding site (AGTGANNNNNNCCACG) 169 bp upstream of the translation start site (Fig. 1b). In order to define the promoter region, we determined the transcription start point (TSP) of the *whiB1* transcript by the 5'-RACE

Fig. 1. Identification of the TSP of the *M. tuberculosis whiB1* gene by 5'-RACE. (a) Mapping of the 5' end of the *M. tuberculosis whiB1* transcript by the 5'-RACE method. Fragments were amplified by PCR using the abridged anchor primer (Invitrogen) in combination with the gene-specific primer *whiB1*/RACE1 (not shown). The resulting amplicon was used as the template for another PCR amplification by using the abridged universal amplification primer (Invitrogen) and gene-specific primer *whiB1*/RACE2, resulting in formation of an amplicon of ~200 bp (lane 1). Lane 2, size markers. DNA sequencing of this fragment identified the TSP. (b) Sequence of the *whiB1* 5'UTR. The TSP (+1) identified by 5'-RACE is indicated by a right-angled arrow. Positions of bases upstream of the TSP are indicated by numbers above the respective bases. The predicted -10 and -35 sequences are indicated by boldface. The putative ribosome-binding site (RBS) and the translation start codon (ATG) are boldfaced and underlined. The putative CRP-binding site is shaded. Horizontal arrows indicate the positions and directions of the primers that were used for PCR amplification of DNA fragments.

technique, as described in Methods. Total RNA was isolated from *M. tuberculosis* in mid-exponential phase (OD₆₀₀ 0.6), and final amplification of dCTP-tailed cDNA was performed using primers AUAP and *whiB1*/RACE2 (Fig. 1b). As shown in Fig. 1(a), PCR amplification of the RACE cDNA products revealed a major band (>200 bp in size) which upon DNA sequencing corresponded to a *whiB1* TSP from a purine base, G, located 109 bp upstream of the translation start codon (Fig. 1b). Analysis of the sequence upstream of this TSP revealed the presence of promoter-like sequences at the -35 (TTGACA) and -10 (AACGAT) positions. The minor band (<100 bp in size) observed in Fig. 1(a) proved by sequence analysis to be an artefactual primer-dimer amplification product.

The CRP-binding consensus sequence in *E. coli* (5'-TGTGA-TCTAGA-TCACA-3') exhibits perfect twofold

sequence symmetry, with the bold-faced bases representing the left and right arms for binding of the active CRP dimer (Berg & von Hippel, 1988; de Crombrughe *et al.*, 1984). The putative CRP-binding site in the *whiB1* 5'UTR (5'-AGTGAGATAGCCACG-3') is located between positions -51 and -66 bp (centred at -58.5 bp) upstream of the TSP, and contains the critical residues, 5'-GTG-3' in the left arm and 5'-CAC-3' in the right arm (Fig. 1b). The left arm contains a near-perfect sequence (AGTGA) and the right arm contains 3/5 matching residues (CCACG).

Transcription of *whiB1* is constitutive and positively regulated by CRP^M

In order to assess the expression of the *whiB1* promoter in *M. tuberculosis*, a DNA fragment carrying the wild-type *whiB1* 5'UTR (spanning positions -214 to +33) was

cloned upstream of a promoterless *lacZ* gene in pSD5B (a mycobacterial promoter probe vector), and the recombinant plasmid (pSD5B-wt*whiB1*) was used to transform *M. tuberculosis*. Promoter activity was determined by β -galactosidase assays with cell lysates of bacilli harvested during different phases of growth (OD₆₀₀ 0.4, 0.8, 2.0 and 3.5, respectively). As shown in Fig. 2(b), the β -galactosidase specific activity resulting from *whiB1* promoter expression at different growth phases remained constant, thus indicating that *whiB1* is expressed constitutively in *M. tuberculosis*. Recently, a similar pattern of *whiB1* expression was observed in *M. tuberculosis* by Geiman *et al.* (2006) by using the RT-PCR method.

To evaluate the role of the putative CRP^M-recognition site in the regulation of *whiB1* expression, we constructed a mutant derivative of the *whiB1* promoter. The nucleotides at the

third and fourth positions of the CRP-binding site in the *whiB1* 5'UTR (Fig. 1b), known to be critical for CRP binding in *E. coli* (Jansen *et al.*, 1987), were replaced, altering the wild-type left arm consensus sequence from AGTGA to AGGTA (Fig. 2a). The mutant promoter DNA fragment was cloned in pSD5B (resulting in the construct pSD5B-mt*whiB1*), and the activities of both the wild-type and mutant *whiB1* promoters were compared in *M. tuberculosis* at OD₆₀₀ 0.4, 0.8, 2.0 and 3.5, as described above. As shown in Fig. 2(b), the *whiB1* promoter with a wild-type CRP-binding site exhibited β -galactosidase activities of ~ 7500 nmol min⁻¹ mg⁻¹ in *M. tuberculosis*. Point mutations in the CRP-binding site resulted in a 3–4-fold reduction in β -galactosidase specific activity to ~ 2000 nmol min⁻¹ mg⁻¹ at each OD₆₀₀ tested (Fig. 2b). These observations indicate that the putative CRP-binding site is required in its intact form for full-level *whiB1* expression in *M. tuberculosis*.

Fig. 2. Base substitutions in the left arm of the putative *M. tuberculosis whiB1* CRP-recognition site and their influence on *whiB1* activity and CRP^M binding. (a) Sequence analysis of the *whiB1* promoter with wild-type and mutant CRP recognition sites. Boldfaced letters represent bases at the -35 and -10 positions. The underlined sequence represents the putative CRP-binding site. Positions of mutations in the CRP-binding site are indicated by asterisks. (b) Effects of base substitutions on *whiB1* promoter specific activity. *M. tuberculosis* bacilli harbouring either pSD5B-wt*whiB1* (wild-type) or pSD5B-mt*whiB1* (mutant) promoter constructs were harvested at different phases of growth (OD₆₀₀ 0.4, 0.8, 2.0 and 3.5). Promoter activity was measured as β -galactosidase specific activity. The values are means $\pm$ SD of three separate assays. (c) SDS-PAGE analysis of overproduced CRP^M as a His₆ fusion protein (left lane). Coomassie-blue-stained gel showing the isolated CRP^M protein (400 pmol) following affinity chromatography. M, molecular mass markers. (d) Interaction of CRP^M with the *whiB1* 5'UTR. Biotin-labelled *whiB1* 5'UTR (lanes 2 and 3) or biotin-labelled *whiB1* 5'UTR with mutations in the putative CRP-binding site (5'-AGTGAgaatagcCCACG-3' to 5'-AGGTAgaatagcCCACG-3') (lane 5) was incubated with CRP^M in the presence of 1 mM cAMP before separation of CRP^M-*whiB1* complexes by gel electrophoresis. As a specificity control, unlabelled competitor DNA was added in 20-fold molar excess (lane 3). Lanes 1 and 4 contain *whiB1* promoter fragments with wild-type and mutant CRP-recognition sequences, respectively, in the reaction mixture lacking CRP^M.

CRP^M regulates expression of *whiB1* by directly binding to the upstream CRP recognition site

Based on the *whiB1* promoter activities in mycobacteria, we examined whether *whiB1* expression was regulated by direct binding of CRP^M to the putative CRP-recognition sequence adjacent to the *whiB1* promoter. We performed EMSA analyses using purified recombinant CRP^M and DNA comprising the *whiB1* promoter region and putative CRP binding site. The target DNA region extended from base -214 to +33 relative to the TSP, and was PCR-amplified using forward primer PwhiB1(F) and biotin-labelled reverse primer PwhiB1(R) (Fig. 1b) as described in Methods. Recombinant CRP^M protein was expressed in *E. coli* as a fusion protein with a His₆ tag at its C-terminus and purified using metal affinity chromatography. Analysis of the purified protein by SDS-PAGE confirmed that the preparation was homogeneous, and CRP^M had an apparent molecular mass of approximately 26 kDa, which is in good agreement with its predicted molecular mass (Fig. 2c).

Fig. 3. Effects of cAMP concentration on CRP^M-*whiB1* complex and expression of *whiB1* in *M. tuberculosis*. (a) Effects of increasing cAMP concentrations on the binding of CRP^M to the *whiB1* 5'UTR. Purified CRP^M was incubated with the different concentrations of cAMP for 30 min on ice, before binding to the *whiB1* 5'UTR. Biotin-labelled *whiB1* 5'UTR (2 nM) was incubated with cAMP-CRP^M (1.5 μM) for 15 min at 37 °C before separation of CRP^M-*whiB1* complexes by gel electrophoresis. The cAMP concentrations used are indicated above the respective lanes. Lane C, control reaction lacking CRP^M. Complex, CRP^M-*whiB1* complex; Free, unbound DNA fragment. (b) Effects of increasing cAMP concentrations on the binding of CRP^M to the *whiB1* 5'UTR with mutations in the CRP-binding site (5'-AGTGA_{gatagc}CCACG-3' to 5'-AGGT_AgatagcCCACG-3'). Biotin-labelled mutant *whiB1* 5'UTR (2 nM) was incubated with cAMP-CRP^M (1.5 μM) for 15 min at 37 °C before separation of CRP^M-*whiB1* complexes by gel electrophoresis. The cAMP concentrations used are indicated above the respective lanes. Free, unbound DNA fragment. (c) Effects of db-cAMP on *whiB1* promoter specific activity. *M. tuberculosis* bacilli harbouring either pSD5B-wt*whiB1* (wild-type) or pSD5B-mt*whiB1* (mutant) promoter constructs were harvested 2 h after exposure to either db-cAMP or butyric acid (BA), as described in Methods. Promoter activity was measured at different phases of growth (OD₆₀₀ 0.4, 0.8, 2.0 and 3.5) as β-galactosidase specific activity. The values are means ± SD of three separate assays.

As shown in Fig. 2(d), CRP^M was able to bind the wild-type *whiB1* 5'UTR with a concentration of 1.5 μM per 2 nM template DNA fragment (Fig. 2d, lane 2). Addition of CRP^M at lower concentrations did not result in sufficient complex formation to be detected by EMSA under these conditions (data not shown). In order to confirm that the binding was specific, the binding reactions were carried out in the presence of a 20 molar excess of unlabelled target DNA. In the presence of competitor, binding was completely abolished (Fig. 2d, lane 3), indicating that CRP^M binds specifically to the *whiB1* 5'UTR. To verify that CRP^M binds to its predicted CRP recognition site in the *whiB1* 5'UTR, we performed the EMSA experiment with a *whiB1* 5'UTR containing a defective CRP-binding site (substitution of the left arm consensus sequence from 5'-AGTGA-3' to 5'-AGGTA-3', Fig. 2a) as the template. As shown in Fig. 2(d), lane 5, CRP^M is unable to bind to the mutant CRP-recognition site in *whiB1* 5'UTR, thus supporting the premise that CRP^M directly regulates the expression of *whiB1* in *M. tuberculosis* by specifically binding to the CRP-recognition site adjacent to the *whiB1* promoter.

Regulation of the expression of *whiB1* by CRP^M requires cAMP

It is well established that both CRP and CRP^M specifically bind to DNA at CRP recognition sites in the presence of cAMP (Bai *et al.*, 2005; Botsford & Harman, 1992; Busby & Buc, 1987; Crasnier, 1996; Kolb *et al.*, 1993; Lawson *et al.*, 2004). To characterize the role of cAMP in CRP^M binding to the *whiB1* 5'UTR, we carried out EMSA with CRP^M and the

whiB1 5'UTR in the presence of a gradient of cAMP concentrations varying from 0.1 μ M to 1 mM (Fig. 3a). Since no complex was observed when cAMP was omitted from the binding reaction, our results demonstrate that the mycobacterial CRP requires cAMP for significant binding to the *whiB1* 5'UTR (Fig. 3a, lane 2). Although binding of CRP^M to the *whiB1* 5'UTR was observed in the presence of cAMP at concentrations as low as 0.1 mM, maximal complex formation required 1 mM cAMP (Fig. 3a). In addition, CRP^M was unable to bind to the mutant CRP-recognition site in the *whiB1* 5'UTR at any concentration of cAMP used, thus indicating that CRP^M binds specifically to the CRP-recognition site in the *whiB1* 5'UTR (Fig. 3b). Our results therefore indicate that the ability of CRP^M to bind the *whiB1* 5'UTR varied directly with the cAMP concentration.

The requirement of cAMP for the binding of CRP^M to the *whiB1* 5'UTR *in vitro* implies that cAMP levels regulate the expression of *whiB1* in bacteria *in vivo*. We therefore analysed the effects of the cAMP analogue db-cAMP on the activities of wild-type and mutant *whiB1* promoters in *M. tuberculosis*. In order to assess early transcriptional effects of cAMP addition rather than late-stage potentially pleiotropic phenomena, *M. tuberculosis* bacilli harbouring either pSD5B-wt*whiB1* or pSD5B-mt*whiB1* were harvested 2 h after exposure to db-cAMP, as described in Methods. Promoter activity was determined by β -galactosidase assays with cell lysates of bacilli harvested after treatment with either db-cAMP or butyric acid at different phases of growth (OD₆₀₀ 0.4, 0.8, 2.0 and 3.5). As shown in Fig. 3(c), at all OD₆₀₀ levels, db-cAMP treatment resulted in a 2–2.5-fold increase in the activity of the wild-type *whiB1* promoter, compared with its activity in bacilli grown in the presence of butyric acid. However, transcription by the mutant *whiB1* promoter essentially remained constant under both these conditions (Fig. 3c). These results thus suggest that *whiB1* expression in *M. tuberculosis* may be controlled by a molecular switch provided by the cellular cAMP levels.

DISCUSSION

The sequenced genome of *M. tuberculosis* contains seven genes belonging to the *whiB* family. Recent studies of these mycobacterial *whiB* paralogues have underscored the importance of several of the WhiB proteins for *M. tuberculosis* physiology. Recently, *S. coelicolor* WhiD, the orthologue of *M. tuberculosis* WhiB3, was shown to bind a [4Fe–4S] cluster (Jakimowicz *et al.*, 2005). In all probability the four cysteine residues, conserved in all but one of the Wbl proteins identified so far, are involved in binding the iron–sulfur cluster. As a consequence of this interaction, it has been postulated that the WhiD protein may play a role in maintaining the redox balance of the cell. The presence of a putative helix–turn–helix motif in the WhiB protein family implies that these proteins may perform a regulatory role as DNA-binding proteins. Recently, Kim *et al.* (2005) proposed that the orthologue of WhiB1 in *Corynebacterium glutamicum*, WhcE, may be involved in the regulation of

genes sensing redox changes at the different stages of cell growth.

Based on a recent report (Rickman *et al.*, 2005), we have chosen to focus on CRP-dependent regulation of the expression of *M. tuberculosis whiB1*. In *E. coli*, the CRP-dependent promoters are grouped into three classes. Class I has a single CRP-binding site, centred at one of the positions –61.5, –71.5, –81.5 or –91.5; class II possesses a CRP-recognition site centred at –41.5, which thus overlaps with the –35 sequence; and class III requires two or more CRP-binding sites in the promoter for full activation (Busby & Ebright, 1999; Ebright, 1993; Ushida & Aiba, 1990). Based on the position of the CRP-recognition sequence –58.5 nt upstream of the TSP, the *M. tuberculosis whiB1* promoter region most resembles the class I CRP-dependent promoters of *E. coli*.

Our study indicates that CRP^M binds to a CRP recognition site upstream of the *M. tuberculosis whiB1* promoter in a cAMP-dependent manner *in vitro*, and that db-cAMP administered exogenously strongly induces *M. tuberculosis whiB1* transcription *in vivo*. The higher activity of the wild-type *whiB1* promoter in comparison to its mutated derivative, lacking a functional CRP-recognition sequence, indicates a requirement for CRP^M binding to drive full-level expression of *whiB1* in *M. tuberculosis*. However, despite the presence of a functional CRP recognition site, we observed constitutive expression of the wild-type promoter through the growth cycle. This may reflect relatively constant intracellular cAMP levels during mycobacterial growth in the glucose-rich OADC-supplemented 7H9 medium. We also observed constitutive expression from the mutant *whiB1* promoter lacking the CRP recognition sequence at levels approximately three- to fourfold lower than those with an intact CRP recognition site, and we postulate that this represents a basal expression level from the σ^{70} -like promoter consensus sequences at the –35 (TTGACA) and –10 positions (AACGAT) in the core *whiB1* promoter.

An earlier study by Rickman *et al.* (2005) found that *whiB1* transcription was reduced in an *M. tuberculosis* mutant lacking an intact Rv3676 gene, which encodes CRP^M. However, the CRP-recognition consensus sequence in the *M. tuberculosis whiB1* 5'UTR lacks perfect twofold symmetry and diverges from the canonical *E. coli* consensus sequence at several positions. Indeed, a recent report which identified 73 CRP binding motifs in *M. tuberculosis* using a Gibbs sampling computational search model did not detect the *whiB1* promoter region as a significant match (Bai *et al.*, 2005). Despite the lack of perfect twofold symmetry and several differences from the *E. coli* consensus sequence, our observations, together with those of Rickman *et al.* (2005), indicate that certain divergent CRP^M-recognition sites such as that of *whiB1* are likely to retain biologically significant activity in *M. tuberculosis*.

cAMP is an important signalling molecule regulating various cellular functions, including virulence factors

from a diverse range of pathogens (Alspaugh *et al.*, 2002; Caler *et al.*, 2000; D'Souza & Heitman, 2001; Gross *et al.*, 2003; Lee *et al.*, 2003; Li *et al.*, 2002). There is little information about the role of cAMP in mycobacteria, despite its presence in both fast- and slow-growing, as well as pathogenic and nonpathogenic, species (Padh & Venkatasubramanian, 1977). The genome sequence of *M. tuberculosis* revealed 15 ORFs that contain a cyclase homology domain (Cole *et al.*, 1998; McCue *et al.*, 2000), which implies that cAMP-mediated signal transduction may be a central and versatile tool that this pathogen may employ to combat multiple environmental challenges. Indeed, in one novel role for cAMP in mycobacterial pathogenesis, *Mycobacterium microti*, a member of the *M. tuberculosis* complex, has been reported to secrete cAMP during infection of macrophages. It is postulated that secreted cAMP may prevent phagolysosome formation and thereby protect bacilli from host-mediated destruction (Lowrie *et al.*, 1975, 1979). Recently, it has been observed that exogenous cAMP induced the expression of several biologically significant genes in *M. bovis* BCG, thus implicating cAMP-mediated regulation of gene expression as an important mechanism in the *M. tuberculosis* complex (Gazdik & McDonough, 2005).

We conclude that *whiB1* is the first gene of the *wbl* family shown to be regulated by cAMP in *M. tuberculosis*. Future studies of the effects of cAMP on *whiB1* expression and subsequent downstream effects mediated by *whiB1* will better define the role of cAMP signalling and Wbl-mediated gene regulation in pathogenic mycobacteria.

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