**Mycobacterium tuberculosis** cAMP Receptor Protein (Rv3676) Differs from the *Escherichia coli* Paradigm in Its cAMP Binding and DNA Binding Properties and Transcription Activation Properties*

The pathogen *Mycobacterium tuberculosis* produces a burst of cAMP upon infection of macrophages. Bacterial cyclic AMP receptor proteins (CRP) are transcription factors that respond to cAMP by binding at target promoters when cAMP concentrations increase. Rv3676 (CRP<sup>MT</sup>) is a CRP family protein that regulates expression of genes (rpfA and whiB1) that are potentially involved in *M. tuberculosis* persistence and/or emergence from the dormant state. Here, the CRP<sup>MT</sup> homodimer is shown to bind two molecules of cAMP (one per protomer) at noninteracting sites. Furthermore, cAMP binding by CRP<sup>MT</sup> was relatively weak, entropy driven, and resulted in a relatively small enhancement in DNA binding. Tandem CRP<sup>MT</sup>-binding sites (CRP1 at −58.5 and CRP2 at −37.5) were identified at the whiB1 promoter (PwhiB1). *In vitro* transcription reactions showed that CRP1 is an activating site and that CRP2, which was only occupied in the presence of cAMP or at high CRP<sup>MT</sup> concentrations in the absence of cAMP, is a repressing site. Binding of CRP<sup>MT</sup> to CRP1 was not essential for open complex formation but was required for transcription activation. Thus, these data suggest that binding of CRP<sup>MT</sup> to the PwhiB1 CRP1 site activates transcription at a step after open complex formation. In contrast, high cAMP concentrations allowed occupation of both CRP1 and CRP2 sites, resulting in inhibition of open complex formation. Thus, *M. tuberculosis* CRP has evolved several distinct characteristics, compared with the *Escherichia coli* CRP paradigm, to allow it to regulate gene expression against a background of high concentrations of cAMP.

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<sup>1</sup> The abbreviations used are: CRP, cyclic AMP receptor protein; α-CTD, RNA polymerase α-subunit C-terminal domain; CRP<sub>αCTD</sub>, *M. tuberculosis* CRP protein encoded by the gene Rv3676; RNAP, RNA polymerase.
The *M. tuberculosis* Rv3676 protein (hereafter CRP<sub>Mt</sub>) is a member of the CRP family (15–17). CRP<sub>Mt</sub> is 32% identical (53% similar) over 189 amino acids to *E. coli* CRP (16). Like CRP in *E. coli*, CRP<sub>Mt</sub> is a global transcriptional regulator because a deletion mutant has altered transcription of a large number of genes (16). Moreover, it is implicated in the virulence of *M. tuberculosis* because the CRP<sub>Mt</sub> mutant is attenuated for growth in mice and macrophages as well as in *in vitro* (16). Polymorphisms in CRP that enhance DNA binding have also occurred in the Bacillus Calmette-Guérin vaccine strain of *Mycobacterium bovis* (18–20) and result in changes in transcription of a number of genes, which, although not contributing to the attenuation of Bacillus Calmette-Guérin, may have been selected by growth in *in vitro* (20).

In the CRP<sub>Mt</sub> mutant, the largest decreases in expression were for the *rpfA* and *whiB1* genes (16). *In vivo* and *in vitro* analyses indicated that CRP<sub>Mt</sub> activates expression of *rpfA* and *whiB1* (16, 17). These are potentially significant observations because *rpfA* encodes a protein that is thought to be involved in reviving dormant bacteria (21), and *whiB1* encodes a Wbl family protein (22). Wbl proteins are found only in actinomycetes and bind redox-sensitive iron-sulfur clusters (23, 24). The mechanism(s) of action of Wbl proteins is still unclear; some have been reported to have protein-disulfide reductase activity (24), and at least one (WhiB3) has been shown to bind DNA (25), consistent with the suggestion that Wbl proteins are transcription factors that might function in the control of developmental processes (22, 26). This latter suggestion raises the possibility that CRP<sub>Mt</sub> in complex with cAMP regulates genes involved in the developmental switch associated with *M. tuberculosis* persistence and/or emergence from the dormant state. However, previous work suggested that although CRP<sub>Mt</sub> binds cAMP, this interaction induces a relatively small enhancement in specific DNA binding (15–17). Thus, there are differences between *E. coli* CRP, where the presence of cAMP enhances specific DNA binding by several orders of magnitude (27), and CRP<sub>Mt</sub>. Hence, the aim of this work was to investigate the interaction between CRP<sub>Mt</sub> and cAMP and determine the mechanism of CRP<sub>Mt</sub>-mediated activation of *whiB1* expression. Here, the following points are shown: (i) CRP<sub>Mt</sub> dimer binds two molecules of cAMP; (ii) unlike *E. coli* CRP, the CRP<sub>Mt</sub> cAMP-binding sites do not interact; (iii) CRP<sub>Mt</sub> binds at two immediately adjacent sites in the *whiB1* promoter; and (iv) occupation of the upstream CRP<sub>Mt</sub>-binding site at low cAMP concentrations activates *whiB1* transcription at a step after open complex formation, whereas occupation of the downstream site at high cAMP concentrations antagonizes activation from the upstream site by preventing open complex formation. In addition, a molecular model based on the *E. coli* CRP structure provides a plausible explanation for the distinctive cAMP binding properties of CRP<sub>Mt</sub>.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—Bacterial strains and plasmids are listed in Table 1. *E. coli* cultures were grown in Luria-Bertani (LB) medium (36) in a 1:5 volume/flask ratio at 37 °C with shaking at 250 rpm, except for *in vivo* transcription experiments where strains were grown in a 1:25 volume/flask ratio. Where required, antibiotics were added to media at the following concentrations: tetracycline 35 μg ml<sup>−1</sup>, kanamycin 50 μg ml<sup>−1</sup>, ampicillin 100 μg ml<sup>−1</sup>. *M. tuberculosis* cultures (100 ml) were grown in 1 l of polycarbonate culture bottles (Techmate) in a Bellco roll-in incubator (2 rpm) at 37 °C in Dubos broth containing 0.05% (v/v) Tween 80 supplemented with 0.2% (v/v) glycerol and 4% Dubos medium albumin. Where required, kanamycin was added at a final concentration of 25 μg ml<sup>−1</sup>. *Mycobacterium smegmatis* was grown to log phase (56 h) in LB medium in a 1:5 volume/flask ratio at 37 °C with shaking at 250 rpm.

**Overproduction and Purification of CRP<sub>Mt</sub>**—The CRP<sub>Mt</sub> (Rv3676) open reading frame was amplified by PCR using primers Myc1746 (5′-CATCATGAAATCTGACGAGATCCTGGCC-3′) and Myc1747 (5′-CATCATATCTGACGACATATTAACCTCGCTCGCGGCGGC-3′) containing engineered EcoRI and XhoI sites, respectively. This fragment was ligated into the corresponding sites of a pET28a derivative, in which the kanamycin resistance gene had been disrupted by the insertion of an ampicillin resistance gene (*bla*). The resulting plasmid (pGS2132) encoded a His<sub>6</sub>-CRP<sub>Mt</sub> fusion protein. The plasmid pGS2132 was moved into *E. coli* strain JRG5876 (BL21DE3 ΔcyaA), for expression of the recombinant protein by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, followed by a further 3-h growth at 37 °C before collecting the bacteria by centrifugation. The bacteria were lysed by resuspending in 20 mM sodium phosphate, pH 7.2, containing 0.5 M NaCl, followed by repeated freeze-thawing and sonication. The lysate was cleared by centrifugation, and the resulting cell-free extract was passed through a nickel-charged Hi-Trap chelating column (GE Healthcare). The recombinant His<sub>6</sub>-CRP<sub>Mt</sub> protein was eluted using an imidazole gradient (0–500 mM in 20 ml). The pooled fractions containing His<sub>6</sub>-CRP<sub>Mt</sub> were dialyzed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>), and 10% (v/v) glycerol was added to the protein before storage at −20 °C. Where indicated the His<sub>6</sub> tag was removed by treatment with the protease thrombin (10 units for 16 h at 4 °C).

**Trypsin Digestion of His<sub>6</sub>-CRP<sub>Mt**—Recombinant His<sub>6</sub>-CRP<sub>Mt</sub> (15 μg) was incubated with 2 mM cAMP or cGMP for 10 min at 37 °C, followed by the addition of a second cyclic nucleotide (2 mM) for 10 min where indicated. The protein was then cleaved with 1 μg of trypsin (Sigma) for up to 10 min at 20 °C, and the reaction was stopped by the addition of 1.3% SDS and heating to 100 °C for 10 min. The resulting fragments were analyzed on a 15% SDS-polyacrylamide gel.

**Isothermal Calorimetry**—Recombinant His<sub>6</sub>-CRP<sub>Mt</sub> was extensively dialyzed against phosphate-buffered saline, and the concentration of protein was determined by SDS-PAGE and amino acid analysis (ion exchange chromatography and ninhydrin detection). The sodium salt of cAMP was dissolved in the dialysate phosphate-buffered saline, and the concentration was determined by UV absorption spectroscopy using the extinction coefficient of ε<sub>260</sub> of 1.23 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup>. All samples were centrifuged prior to the titrations. The titration calorimetry measurements were performed using a MicroCal VP-ITC (MicroCal LLC, Northampton, MA). The isothermal calorim-
Transfer of Plasmids, Preparation of Cell-free Extracts, and Assay for β-Galactosidase in M. tuberculosis—These were carried out as described previously (16). β-Galactosidase assays on log phase cultures (A600 nm ~ 0.5) were done according to Miller (38). Three independent cultures were analyzed for each strain.

5′ Rapid Amplification of cDNA Ends—5′ Rapid amplification of cDNA ends was performed using the 5′ rapid amplification of cDNA ends system from Invitrogen according to the manufacturer’s instructions. DNA-free RNA (5 µg) from M. tuberculosis H37Rv was reverse-transcribed with GSP1 (5′-TACGCGCTTTCTGTCG-3′) using Superscript II reverse transcriptase. The cDNA was purified on a SNAP column and tailed with dCTP using terminal deoxynucleotidyltransferase. The tailed cDNA was amplified using Platinum® Taq with primers GSP2 (5′-GGCCGCTCTGTTCGGATCTG-3′) and AAP (5′-GGCCACCGGTCGAATGAGCAAGCGGAGG-3′). The product was visualized on a 1.5% agarose gel, and a band of ~300 bp was excised and sequenced.

Construction of Reporter Gene Plasmids Using the Upstream Region of whiB1—The region of the DNA sequence upstream of whiB1 was generated by PCR from E. coli strains carrying a copy of the T7 RNAP under the control of the IPTG-inducible lacUV5 promoter. The cDNA was purified on a SNAP column and tailed with dCTP using terminal deoxynucleotidyltransferase. The tailed cDNA was amplified using Platinum® Taq with primers GSP2 (5′-GGCCGCTCTGTTCGGATCTG-3′) and AAP (5′-GGCCACCGGTCGAATGAGCAAGCGGAGG-3′). The product was visualized on a 1.5% agarose gel, and a band of ~300 bp was excised and sequenced.

Mutagenesis of CRP-binding Site in Plasmid pRB142—This was performed using the Stratagene QuickChange mutagenesis kit. To mutagenize CRP1 (AGTGAGATAGCCCACG to AGTGAGATAGCCATCG), the primers were used Myc896 (5′-AACGAGATCGCAGATGAGCAGCGG-3′) and Myc487 (5′-GCTCTAGAGCGGATCTGCTAC-3′). The fragment was ligated into the XbaI and HindIII sites of the polylinker in the lacZ transcriptional reporter plasmid pEJ414 (34) to make pRB142 (PwhiB1). This construct was verified by DNA sequencing.

M. tuberculosis strains

| Strain or plasmid | Relevant characteristics | Source or Ref. |
|------------------|-------------------------|---------------|
| H37Rv            | Wild-type virulent strain | 30            |
| ΔRv3676         | H37Rv, deletion of Rv3676 (CRPmt) | 16            |
| H37Rv/pRB142    | H37Rv, with whiB1-lacZ reporter plasmid pRB142 | This work |
| H37Rv/pRB143    | H37Rv, with whiB1-lacZ reporter plasmid pRB143 | This work |
| H37Rv/pRB144    | H37Rv, with whiB1-lacZ reporter plasmid pRB144 | This work |
| H37Rv/pRB145    | H37Rv, with whiB1-lacZ reporter plasmid pRB145 | This work |
| H37Rv/pRB146    | H37Rv, with whiB1-lacZ reporter plasmid pRB146 | This work |
| ΔRv3676/pRB142  | ΔRv3676, with whiB1-lacZ reporter plasmid pRB142 | This work |

M. smegmatis strains

| Strain or plasmid | Relevant characteristics | Source or Ref. |
|------------------|-------------------------|---------------|
| JRG5875         | Source of RNAP | 31            |
| pCR4Blunt-TOPO   | General cloning vector for blunt-ended PCR products; ApR, KanR | Invitrogen |
| pET28a          | His6 tag overexpression vector; KanR | Novagen |
| pRW50           | lacZ transcriptional reporter plasmid; TetR | 32            |
| pRS485          | Expression vector with an IPTG-inducible promoter; ApR | 33            |
| pGS1645         | pRS485 containing Rv3676 gene | This work |
| pGS2060         | pCR4Blunt-TOPO containing the region upstream of whiB1 | This work |
| pEJ414          | A derivative of galP1 with a consensus CRP-binding site centered at position ~37.5 bp | 34            |
| pGS2061         | pET28a derivative encoding His6-CRPmt fusion protein; ApR | This work |
| pGS2062         | pCR4Blunt-TOPO containing the 285-bp region upstream of whiB1 | This work |
| pGS2063         | As pGS2060 but with CRPmt site 1 altered to AGTTAGATAGCCACCG | This work |
| pGS2065         | As pGS2060 but with CRPmt site 1 altered to CCAACAACATATGGAC | This work |
| pGS2067         | As pGS2060 but with CRPmt site 2 altered | This work |

M. tuberculosis shuttle plasmids

| Strain or plasmid | Relevant characteristics | Source or Ref. |
|------------------|-------------------------|---------------|
| pEJ414          | lacZ transcriptional reporter plasmid; KanR | E. O. Davis (35) |
| pRB142          | pEJ414 derivative containing transcriptional fusion of whiB1 upstream region with lacZ | This work |
| pRB143          | pRB142 with mutated CRP1 | This work |
| pRB144          | pRB142 with mutated CRP2 | This work |
| pRB145          | pRB142 with mutated CRP1 and CRP2 | This work |
| pRB146          | pRB142 with improved CRP2 | This work |
Characterization of CRP\textsuperscript{Mt}

AACAC-3’) and Myc899 (5’-GTGTTACGTAAGCGCGT- TGGCATATCTACTCGGATCTCGT-3’) to generate pRB143. To mutagenize CRP\textsuperscript{2} (CGTAAACTATTGACA to CGACTATTGACA), the primers used were Myc900 (5’-TAGCCACCCGGCTTACAACTGATTGACCTGCTG-3’) and Myc901 (5’-CAGGCTCAACAGATGCCATGCTATCA- CATTGACA), the primers used were Myc963 (5’-CGCCAGATTAG- ATGCCACCGCTTACAACTGATTGACCTGCTG-3’) to generate pRB144. To mutagenize both CRP1 and CRP2, the primers used were Myc964 (5’-TAGCCACCGGCTTACAACTGATTGACCTGCTG-3’) and Myc965 (5’-CAACAGATGCTCAACAGATGCCATGCTATCA- CATTGACA), the primers used were Myc990 (5’-GACA CACGCGCTTACAACTGATTGACCTGCTG-3’) and Myc991 (5’-GACACTATTGACA), the primers used were Myc155 was disrupted by passage through a French pressure cell. The lysate was then centrifuged and subjected to ammonium sulfate precipitation. The resulting cytoplasmic extract was dialyzed against RNAP buffer (50 mM Tris–Cl, pH 8.0, 10 μM ZnSO\textsubscript{4}, 1 mM EDTA, 10 mM MgCl\textsubscript{2}, and 20% glycerol) containing 10 mM KCl, before loading onto a 5-ml HiTrap heparin column (GE Healthcare). Elution was achieved by applying a linear gradient of 0.01–1 M KCl in RNAP buffer, and the fractions containing RNAP, as determined by SDS-PAGE, were pooled. Dialysis and purification were repeated twice, using a 1-ml HiTrap SP HP cation exchange column followed by a 1-ml HiTrap Q HP anion exchange column (GE Healthcare). Fractions containing enriched holo-RNAP, as determined by SDS-PAGE, were desalted into RNAP buffer containing 10 mM KCl and concentrated 10-fold using a Vivaspin concentrator (molecular mass cutoff of 5 kDa; Sartorius). The resulting RNAP was then tested in \textit{in vitro} transcription assays (not shown) and stored in 25% glycerol at ~80 °C.

For \textit{in vitro} transcription reactions, 0.1–1 kb markers were prepared using Perfect RNA Marker template mix (Novagen). A 20-μl reaction containing 0.75 μg of RNA template mix, 80 mM HEPES, pH 7.5, 12 mM MgCl\textsubscript{2}, 10 mM NaCl, 10 mM dithiothreitol, 2 mM ATP, 2 mM GTP, 2 mM CTP, 0.1 mM UTP, 5 μCi of [α-32P]UTP (800 Ci mmol\textsuperscript{-1}, PerkinElmer Life Sciences), 20 units of RiboLock RNase inhibitor (Fermentas), and 50 units of T7 RNAP (Novagen), was incubated for 1 h at 37 °C, before storing at ~20 °C. Markers from ~2 ng of template were used for gel calibration.

The 285-bp region upstream of the \textit{whiB1} gene and the corresponding regions with the altered CRP\textsuperscript{Mt}-binding sites were excised from plasmids pGS2060, pGS2061, p2225, and p2227 using restriction enzymes XbaI and HindIII. These DNA fragments (0.2 pmol) were incubated for 15 min at 37 °C in a 21-μl reaction volume containing 40 mM Tris–Cl, pH 8.0, 10 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 75 mM KCl, 0.1 mM EDTA, 5% glycerol, 250 μg ml\textsuperscript{-1} bovine serum albumin, 0–2 mM cAMP, 2 pmol of \textit{M. smegmatis} CRP\textsuperscript{Mt}, and 0–20 μM CRP\textsuperscript{Mt}. Transcription was initiated by the addition of 4 μl of a solution containing UTP at 50 μM; ATP, CTP, and GTP at 1 mM; and 2.5 μCi of [α-32P]UTP (800 Ci mmol\textsuperscript{-1}, PerkinElmer Life Sciences), followed by incubation for 15 min at 37 °C. Reactions were terminated by the addition of 25 μl of Stop/Loading dye solution (95% formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol) containing 0.1–1-kb markers from ~9.5 ng of template as a loading control. Samples (10 μl) of each reaction were loaded onto a 6% acrylamide gel and analyzed by autoradiography. Autoradiographs were quantified by ImageMaster software (GE Healthcare).

For permanganate footprinting, the \textit{whiB1} promoter fragment was prepared as for electrophoretic mobility shift assay, except that the opposite strand was end-labeled with [α-32P]DCTP. The resulting radiolabeled DNA (~20 ng) was incubated at 20 °C for 5 min in a reaction containing 40 mM Tris–Cl, pH 8.0, 10 mM MgCl\textsubscript{2}, 75 mM KCl, 0.1 mM EDTA, 5% glycerol, 250 μg ml\textsuperscript{-1} bovine serum albumin, 0.1 mM GTP, 0.1 mM UTP, 0–2 mM cAMP, and 0–20 μM His-tagged CRP\textsuperscript{Mt}. \textit{M. smegmatis} RNAP (2 pmol) was added and incubation continued at 37 °C for 15 min. KMnO\textsubscript{4} (1 mM) was added for 10 min at 37 °C, and the reactions were stopped by the addition of 200 μl of 0.3 M sodium acetate, pH 5.2, containing 20 mM EDTA, fol-
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A

1 min 10 min 10 min 10 min

B

Time (min)

0 20 40 60 80 100

coefficient of CAMP

kcal/mole of CAMP

molar ratio

0 0.5 1.5 2.5

FIGURE 1. Characterization of CAMP binding by CRPMt. A, digestion of CRPMt (15 μg) by trypsin (1 μg) in the
presence and absence of cAMP or cGMP (2 μM). The composition of the reaction mixtures is indicated above each lane of typical Coomassie Blue-stained SDS-polyacrylamide gels. For lanes 2, 3, 12, and 13, the reactions were incubated at 37 °C for 1 min. For all other lanes, the reactions were incubated at 37 °C for 10 min. Lanes 1, 8, and 16 are molecular mass markers; the sizes (kDa) of the relevant markers are shown on the left (the full set is 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa, from top to bottom). CRPMt migrates just above the 25-kDa marker. Lane 23 shows a reaction in which CRPMt was preincubated for 10 min with cGMP before adding cAMP and trypsin, indicated by cAMP then cGMP. Lane 24 shows a reaction in which CRPMt was preincubated for 10 min with cAMP before adding cGMP and trypsin, indicated by cAMP then cGMP. B, analysis of CAMP binding by isothermal calorimetry. The upper panel shows the raw binding heats. Integrations of these peaks with respect to time and correction to a per mol basis yield the binding isotherm shown in the lower panel (squares). Also shown in the lower panel (triangles) are the heats of ligand dilution.

RESULTS

Cyclic AMP Binding at Two Independent Sites Enhances CRPMt-DNA Interactions—Whereas E. coli CRP is very responsive to cAMP, exhibiting nonspecific low affinity DNA binding in the absence of cAMP (27), previous reports of the effects of cAMP on the properties of CRPMt have been equivocal. Rickman et al. (16) and Bai et al. (15) found significant binding of CRPMt to target DNA in the absence of cAMP and only marginal enhancement upon addition of up to 0.1 mM cAMP. In contrast, Agarwal et al. (17) failed to detect DNA binding in the absence of cAMP, but binding was observed in the presence of 1 mM cAMP. Others have shown that incubation with cAMP alters intrinsic tryptophan fluorescence (41) and the polypeptide profiles obtained when CRPMt is digested with trypsin (15), implying that cAMP causes conformational changes in CRPMt. To investigate further, we have isolated recombinant His8-tagged CRPMt by overproduction in a cyaA mutant of E. coli, which is unable to synthesize cAMP. This cAMP-free CRPMt protein was then used to determine the polypeptide profiles obtained after digestion of CRPMt with trypsin in the absence and presence of cAMP (Fig. 1A, lanes 2–7). In contrast to E. coli CRP, which is relatively resistant to tryptic cleavage in the absence of cAMP (42), CRPMt was readily digested by trypsin, yielding a major polypeptide of molecular mass ~16 kDa, as estimated by SDS-PAGE (Fig. 1A). In the presence of cAMP, the protein was more resistant to proteolysis, and a major polypeptide of molecular mass ~15 kDa was obtained. In the presence of cGMP, the sensitivity of CRPMt to trypsin was similar to that observed in the absence of cAMP, although a different digestion pattern, which included both major polypeptides (~16 and ~15 kDa) observed in the absence and presence of cAMP, was obtained suggesting that cGMP is bound by CRPMt with concomitant changes in conformation that are different from those invoked by cAMP (Fig. 1A, lanes 10–15). Significantly, addition of cAMP after precubation with cGMP for 10 min, or vice versa, resulted in a limited proteolysis pattern identical to that obtained with cAMP alone indicating that cAMP is the preferred ligand (Fig. 1A, lanes 20–24). To ensure that the presence of the His8 tag was not influencing the interaction of CRPMt with cAMP, the His8 tag was removed by thrombin cleavage, and the partial proteolysis experiments were repeated. This showed that untagged CRPMt exhibited the same behavior as the tagged protein in the absence (supplemental Fig. S1, lanes 3 and 6) and presence (supplemental Fig. S1, lanes 4 and 7) of cAMP suggesting that the His8 tag was not affecting the gross conformational changes induced by cAMP binding. Therefore, the His8-tagged form of CRPMt was considered suitable for further ligand binding studies.

Isothermal calorimetry was used to determine the stoichiometry and affinity of cAMP binding to His8-CRPMt. A typical
titration is shown in Fig. 1B. The data yield good nonlinear least squares fitting to a single set of identical binding sites model and are consistent with each protomer of the CRP Mt dimer binding one cAMP molecule with relatively weak ($K_a \sim 1.7 \times 10^4 \text{ M}^{-1}$) affinity. Furthermore, the binding of cAMP is an endothermic reaction ($\Delta G_b = -23.7 \text{ kJ mol}^{-1}$) with a positive binding enthalpy ($\Delta H_b \sim 30.7 \text{ kJ mol}^{-1}$). Therefore, the entropy change $\Delta S_b$ is $\sim 54.4 \text{ kJ mol}^{-1} \text{ K}^{-1}$, and hence cAMP binding is entropically driven. Chemical cross-linking showed that CRP Mt is a dimer (not shown), and thus the data indicate that unlike the E. coli CRP dimer, the two cAMP-binding sites in the CRP Mt dimer are independent.

The effect of cAMP binding on the ability of CRP Mt to bind DNA in vivo was tested in the heterologous host E. coli because M. tuberculosis has 17 predicted adenyl cyclase proteins, and E. coli has only one, CyaA; and thus it is possible to simply create a cAMP-free background for these experiments. The parent E. coli strain was a crp lac double mutant into which a cyaA mutation was introduced. The readout (β-galactosidase activity) from the simple CRP-repressed promoter CGalΔ4, which contains a consensus CRP site that is recognized by CRP Mt located such that occupation of this site occludes the promoter (18, 34), was used as a measure of the DNA binding activity of CRP Mt as shown previously by Spreadbury et al. (18). In the CyaA+ and CyaA− strains containing the vector (ptac85), transcription from the reporter was similar (761 ± 24 and 722 ± 25 Miller units, respectively, $n = 5$). However, in the presence of the CRP Mt expression plasmid (pGS1645), reporter transcription was ~60% lower (323 ± 86 Miller units, $n = 5$) in the Cya− strain compared with that observed in the absence of CRP Mt, consistent with the observations of Spreadbury et al. (18). However, in the cyaA mutant, which is unable to synthesize cAMP, the readout from the reporter in the presence of CRP Mt increased by ~2-fold (613 ± 20 Miller units, $n = 5$) compared with the Cya+ strain to reach ~80% of the activity observed in the absence of CRP Mt. These data are consistent with cAMP enhancing DNA binding of CRP Mt in vivo.

To investigate the effect of cAMP on CRP Mt DNA binding in vitro, preliminary electrophoretic mobility shift assays were used to show that CRP Mt formed at least two complexes at the whiB1 promoter in the absence and presence of cAMP but that DNA binding was enhanced in the presence of cAMP (supplemental Fig. S2A). Furthermore, in cAMP titration experiments, CRP Mt binding to the whiB1 promoter was enhanced when the cAMP concentration exceeded 0.05 mM (not shown), consistent with the isothermal calorimetry experiments. Moreover, the presence of the His$_8$ tag did not significantly affect DNA binding by CRP Mt in the absence (not shown) and presence of cAMP (supplemental Fig. S2B). Thus, the His$_8$-tagged form was used in further DNA binding studies.

In summary, the work described above shows the following: (i) that CRP Mt binds two cAMP molecules per dimer (one per subunit); (ii) that the cAMP-binding sites act independently; and (iii) that cAMP binding induces conformational changes in the CRP Mt dimer that enhance specific DNA binding in vitro and in vivo.

**whiB1 Promoter Contains Two CRP Mt-binding Sites**—The whiB1 gene encodes a Wbl (WhiB-like) protein. These proteins have iron-sulfur clusters and are found only in actinomycetes (26) where they are thought to function as transcription factors and/or as protein-disulfide reductases. The whiB1 transcript was less abundant in a CRP Mt mutant (16), implying that CRP Mt activates whiB1 expression, which was confirmed using a whiB1-lacZ fusion (17). Rapid amplification of cDNA ends was used to confirm that the whiB1 transcript start was located at 109 or 110 bp upstream of the translational start as reported by Agarwal et al. (17) (data not shown). The electrophoretic mobility shift assays (supplemental Fig. S2A) suggested the presence of more than one CRP Mt-binding site in the whiB1 promoter. Inspection of the whiB1 promoter region indicated the presence of two potential CRP Mt-binding sites upstream of the transcript start (Fig. 2). The first site (CRP1) located at −58.5 relative to the transcript start matches the proposed CRP Mt consensus (NGTGNANNNNNCACA) of Rickman et al. (16) in seven of the eight defined bases (Fig. 2). The second potential site (CRP2) is a poorer match to the consensus (six of the eight defined bases are matched) and is located at −37.5 relative to the transcript start. The CRP1 site has previously been implicated in CRP Mt-dependent activation of whiB1 expression (17).

Dnase I footprinting showed that both CRP1 and CRP2 sites in the whiB1 promoter were recognized by CRP Mt and that binding to both sites was enhanced in the presence of cAMP (Fig. 3A). Titration of the whiB1 promoter with increasing concentrations of CRP Mt showed that the CRP1 site (−70 to −51) was occupied before the CRP2 site (−50 to −29) (Fig. 3A). Furthermore, mutation of CRP1 (indicated by lowercase letters, AGTGAAGATAGGACACG to AGTtGAAGATAGGACACG) or CRP2 (CGTTAACCATTGACCGCATGTTGACGCGGACACACAC) inhibited binding of CRP Mt to these sites (Fig. 3B). Inactivation of CRP1 also impaired, but did not abolish, binding to CRP2 (Fig. 3B, compare lane 5 with 6). Thus, it was concluded: (i) the whiB1 promoter possesses tandem CRP Mt sites, (ii) binding to these sites is enhanced in the presence of cAMP; and (iii) occupation of CRP2 is improved by occupation of CRP1.

**Both CRP Mt-binding Sites in the whiB1 Promoter Are Functional**—The Dnase I footprinting studies indicated that there are two CRP Mt sites in the whiB1 promoter. The function-
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**Figure 3. Identification of two CRP<sup>Mt</sup>-binding sites within P<sub>whiB1</sub>**. A, whiB1 promoter (P<sub>whiB1</sub>) has two CRP<sup>Mt</sup>-binding sites. Lanes 2–6 show reactions in the presence of 2 mM cAMP; lanes 7–11 show reactions in the absence of cAMP. Lane 1 shows no CRP<sup>Mt</sup>; lanes 2 and 7 show 2.5 μM CRP<sup>Mt</sup>; lanes 3 and 8 show 5 μM CRP<sup>Mt</sup>; lanes 4 and 9 show 10 μM CRP<sup>Mt</sup>; lanes 5 and 10 show 25 μM CRP<sup>Mt</sup>; lanes 6 and 11 show 50 μM CRP<sup>Mt</sup>; lane 12 shows Maxam and Gilbert G track. WT, wild type. B, mutation of P<sub>whiB1</sub> CRP1 impairs binding of CRP<sup>Mt</sup> to CRP2. All reactions contained cAMP (2 mM). Lanes 1–4 show reactions of the indicated promoter variants in the absence of CRP<sup>Mt</sup> as follows: −1, CRP1 site impaired (AGTGAAGATCCACG to AGTCAAGATCCACG); −2, CRP2 site impaired (CGTAACACTATTGACA to CCAACACTATTGACA), and −12, both sites impaired. Lanes 5–8, DNase I footprints in the presence of 50 μM CRP<sup>Mt</sup>. Lanes 9–12, Maxam and Gilbert G tracks. The locations of the CRP1 and CRP2 sites (see Fig. 2) are indicated by boxes. The footprints shown are typical of at least three experiments.

The presence of cAMP, mutation of both CRP<sup>Mt</sup> sites resulted in transcription similar to that of the unaltered promoter in the absence of CRP<sup>Mt</sup> (Fig. 4, C and D, lanes 8 and 16). In the absence of cAMP, transcription from the whiB1 promoter with both CRP1 and CRP2 impaired was lower than from the unaltered promoter in the absence of CRP<sup>Mt</sup>, suggesting that in the absence of cAMP there is still some unproductive interaction between CRP<sup>Mt</sup> and the altered whiB1 promoter despite the impairment of both CRP-binding sites (Fig. 4, C and D, lanes 4 and 12). Note that transcription in the absence of CRP<sup>Mt</sup> for all the altered promoters was the same as that for the unaltered whiB1 promoter, indicating that the changes to the sequences of the CRP sites had not affected the basal level of transcription (data not shown).

The in vitro transcription experiments showed that CRP<sup>Mt</sup> acts as both an activator (at low concentrations) and repressor (at high concentrations) of whiB1 expression. Permanganate footprinting to detect open complex formation showed the presence of quantitatively similar distortions of DNA at nucleotide T −8 in the whiB1 promoter mediated by RNAP in the presence or absence of 2.5 μM CRP<sup>Mt</sup> (Fig. 5A, lanes 3, 4, 8, and 9). This evidence suggests that CRP<sup>Mt</sup>-mediated activation of whiB1 expression probably occurs after open complex formation, because in the absence of CRP<sup>Mt</sup> transcription from the whiB1 promoter is low (Fig. 4A, compare lanes 3 and 4). In the presence of 20 μM CRP<sup>Mt</sup>, the open complex was not detected (Fig. 5A, lanes 5 and 10) indicating that higher concentrations of CRP<sup>Mt</sup> inhibit whiB1 expression at a point before open complex formation, probably by inhibiting RNAP binding.

DNase I footprinting of the whiB1 promoter in the presence of M. smegmatis RNAP and activating concentrations of CRP<sup>Mt</sup> (2.5 μM) showed that RNAP could bind at the promoter when

The conclusion was supported by in vitro transcription reactions with whiB1 promoters carrying mutations in CRP1 and/or CRP2 (Fig. 4C). At a low CRP<sup>Mt</sup> concentration (2.5 μM) in the absence of cAMP, the footprinting evidence indicates that CRP1 will be occupied at the wild-type promoter. Under these conditions, mutation of the CRP1 site decreased transcription of whiB1 (Fig. 4, C and D, lanes 1 and 2). Under the same conditions, mutation of CRP2 slightly enhanced whiB1 transcription (Fig. 4, C and D, lanes 1 and 3). A similar pattern was observed in the presence of cAMP (Fig. 4, C and D, lanes 5–7). At a higher CRP<sup>Mt</sup> concentration (20 μM), the footprinting indicates that both CRP1 and CRP2 will be occupied. Under these conditions, IMPAIRMENT of CRP1 had little effect on transcription compared with the wild-type promoter, i.e. transcription remained low (Fig. 4, C and D, lanes 9 and 10 and lanes 13 and 14). However, impairment of CRP2 enhanced transcription of whiB1 compared with the wild-type promoter under these conditions (Fig. 4, C and D, lanes 9 and 11, and lanes 13 and 15). Hence, these observations suggest that occupation of CRP2 is sufficient to repress basal transcription from P<sub>whiB1</sub>. In the presence of cAMP, mutation of both CRP<sup>Mt</sup> sites resulted in transcription similar to that of the unaltered promoter in the absence of CRP<sup>Mt</sup> (Fig. 4, C and D, lanes 8 and 16). In the absence of cAMP, transcription of CRP1 and CRP2 impaired was lower than from the unaltered promoter in the absence of CRP<sup>Mt</sup>, suggesting that in the absence of cAMP there is still some unproductive interaction between CRP<sup>Mt</sup> and the altered whiB1 promoter despite the impairment of both CRP-binding sites (Fig. 4, C and D, lanes 4 and 12). Note that transcription in the absence of CRP<sup>Mt</sup> for all the altered promoters was the same as that for the unaltered whiB1 promoter, indicating that the changes to the sequences of the CRP sites had not affected the basal level of transcription (data not shown).
the CRP1 site was occupied and that this was accompanied by the appearance of an RNAP-dependent hypersensitive site at position −34, which is within the CRP2 site (Fig. 5B). The presence of the hypersensitive site is attributed to docking of the C-terminal domain of the RNAP α-subunit downstream of CRP Mt bound at CRP1.

To determine whether the effects of CRP Mt on whiB1 transcription observed in vitro were also apparent in vivo, a promoter fusion containing DNA from −187 to 129 relative to the transcript start was fused upstream of a lac reporter gene, and transcription was estimated in M. tuberculosis wild-type H37Rv and an isogenic Rv3676 (crp) mutant (Fig. 6). Under exponential growth conditions, expression of whiB1 was decreased by −3-fold in the CRP Mt mutant (ΔRv3676), consistent with CRP Mt-dependent activation. An −5-fold reduction in expression was observed when the CRP1 site was disabled, reflecting the absence of activation from CRP1 but retention of repression from CRP2. Accordingly, mutation of CRP2, without disrupting the −35 element (underlined) of the whiB1 promoter (CGTAAACATTTGACA to CcaAACATTTGACA), resulted in a small but reproducible increase in whiB1 expression. Similarly, improvement of the CRP2 site (CGTAAACATTTGACA to CGTGCACATTTGACA) caused a reproducible decrease in whiB1 expression. Disabling both CRP1 and CRP2 lowered β-galactosidase activities by an amount similar to that observed using the unaltered promoter in the crp mutant. The in vivo data correlated well with the in vitro transcription data as shown in Fig. 4, C and D. Thus, when 2.5 μM CRP Mt was used in the in vitro transcription reactions (Fig. 4D), the fold changes in transcription upon impairment of CRP-binding sites compared with the unaltered promoter were as follows: −3.5 to −2.4 when CRP1 was impaired; +1.2 to +1.6 when CRP2 was impaired; and −2.6 to −2.0 when both CRP1 and CRP2 were impaired. These values are similar to those obtained for transcription in vivo (Fig. 6) as follows: −4.6 when CRP1 was impaired; +1.2 when CRP2 was impaired; and −2.0 when CRP1 and CRP2 were impaired. Hence, the in vitro and in vivo experiments are consistent with a mechanism in which occupation of CRP1 enhances whiB1 expression, whereas occupation of both CRP1 and CRP2 or of CRP2 alone represses whiB1 expression in M. tuberculosis.

**DISCUSSION**

The work described here shows that CRP Mt is a homodimeric protein with one cAMP-binding site per promoter. This conclusion is substantiated by the crystal structure of CRP Mt bound to cAMP that was published during the review of this manuscript (43). For E. coli CRP, CAMP binding is cooperative; the first binding event is exothermic and the second is endothermic, and the sensory domain binding sites are satu-
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Rate of micromolar concentrations of cAMP (44). This is not the case for CRPMt, where the cAMP-binding sites are independent. The cAMP binding parameters for CRPMt are similar to those for cGMP binding to E. coli CRP, except that the thermodynamic properties of these interactions are opposite; CRPMt cAMP binding has a positive enthalpy, whereas those for cGMP binding to E. coli CRP have been reported to be rather high (49), being 100–200-fold greater than for CRPMt. The high intracellular concentration of cAMP in M. tuberculosis occurs at higher levels than those in E. coli. Indeed, cAMP concentrations in mycobacteria have been reported to be rather high (49), being ~100–200-fold greater than for E. coli. The high intracellular concentration of cAMP in M. tuberculosis is consistent with the numerous adenylyl cyclases synthesizing cAMP. This and the reported increase in cAMP levels that occurs after infection of

In FIGURE 5, M. tuberculosis CRP activates whiB1 transcription after open complex formation. A, permanganate footprints were obtained with PwhiB1 in the presence and absence of M. smegmatis RNAP and CRPMt. Lanes 1 and 6 show CRPMt 2.5 μM only; lanes 2 and 7 show CRPMt 20 μM only; lanes 3 and 8 show RNAP only; lanes 4 and 9 show RNAP plus CRPMt 2.5 μM; lanes 5 and 10 show RNAP plus CRPMt 20 μM; lanes 11 shows Maxam and Gilbert G track. Lanes 1–5 show reactions in the absence of cAMP; lanes 6–10 show reactions in the presence of cAMP (2 μM). The location of the −10 element is indicated. B, DNase I footprint of PwhiB1 in the presence of an activating concentration of CRPMt (2.5 μM) and RNAP. Lanes 1 and 2 show no protein; lanes 3 and 4 show CRPMt; lanes 5 and 6 show CRPMt plus RNAP; lane 7 shows Maxam and Gilbert G track. The locations of the CRP1 (protected) and CRP2 (unprotected) sites are indicated by filled rectangles, as is the region of protection afforded by RNAP. The location of the −10 element is also marked. The hypersensitive site within CRP2 that appears in the presence of RNAP is indicated by the arrow. The footprints shown are typical of at least three experiments.

Comparison of the cAMP binding pockets of CRPMt (44) and E. coli CRP (46, 47) reveals that most of the side chains that contact cAMP are either conserved or are conservatively substituted. The significant difference between the two proteins, in the context of the independent cAMP binding of CRPMt compared with the cooperative cAMP binding of E. coli CRP, is the substitution of Ser-128 in E. coli CRP by Asn in CRPMt. Ser-128 makes a cross-subunit contact with cAMP in E. coli CRP; i.e. Ser-128 of subunit B interacts with cAMP bound at subunit A N7 of cAMP, and the OD1 atom can accept a hydrogen bond from one of the N6 donor hydrogen atoms (Fig. 7B). This pattern of interactions removes the possibility of the side chain of Asn-135 donating a hydrogen bond to the main chain carbonyl of residue 131 of subunit B. For this reason, replacement of the Ser by Asn at this position uncouples the cAMP-binding sites in CRPMt. Consistent with this interpretation, the substitution of Ser-128 by Ala in E. coli CRP abolishes cooperative cAMP binding (48).

Although cAMP enhanced binding of recombinant CRPMt to target DNA, this enhancement was not comparable with that observed with E. coli CRP, where DNA-binding affinity is enhanced by several orders of magnitude in the presence of 0.1 mM cAMP, allowing specific DNA binding at nanomolar concentrations (27, 48). For CRPMt, a much less significant enhancement of DNA binding was observed, and higher concentrations of cAMP compared with E. coli CRP were required. This may point to meaningful physiological changes in cAMP concentration in M. tuberculosis occurring at higher levels than those in E. coli. Indeed, cAMP concentrations in mycobacteria have been reported to be rather high (49), being ~100–200-fold greater than for E. coli. The high intracellular concentration of cAMP in M. tuberculosis is consistent with the numerous adenylyl cyclases synthesizing cAMP. This and the reported increase in cAMP levels that occurs after infection of
macrophages by pathogenic mycobacteria (5–7) point to cAMP being an important signaling molecule in infection. The evidence presented here suggests that, perhaps as a consequence of the high intracellular concentrations of cAMP in M. tuberculosis, CRP\textsuperscript{Mt} has evolved a different mode of interaction with cAMP compared with the E. coli paradigm, involving low affinity binding of cAMP to independent sites. Nevertheless, the response of CRP\textsuperscript{Mt} to cAMP was very significant for expression of \textit{whiB1}.

Several different classes of regulated bacterial promoters have been identified based on the locations of the binding sites for transcription activators. Promoters dependent on transcription factors bound at or close to -61 are known as class I promoters (Fig. 8). At these promoters a specific region, known as activating region 1 (AR1), of the transcription factor interacts with the C-terminal domain of the \(\alpha\)-subunit (\(\alpha\)-CTD) of RNAP to activate transcription. A common alternative architecture is that of the class II promoters, in which the transcription factor binds to a site that overlaps the core -35 element of the promoter. At class II promoters multiple interactions between the transcription factor and RNAP are possible, including an AR1 interaction with \(\alpha\)-CTD, an AR2 interaction with the RNAP \(\alpha\)-subunit N-terminal domain, and interaction between AR3 and the \(\sigma\)-subunit of RNAP (Fig. 8). Class III promoters have transcription factors bound in tandem making both class I- and class II-type interactions with RNAP (Fig. 8). The experiments described here and elsewhere show that expression of \textit{whiB1} is dependent on CRP\textsuperscript{Mt} and that this requires a CRP\textsuperscript{Mt-binding site centered 58.5 bp upstream of the transcript start, a class I location (16, 17). However, it is now shown that there is a second, lower affinity, negatively acting CRP\textsuperscript{Mt}-binding site (CRP2 centered at -37.5) located immediately downstream of CRP1 that has significant implications for cAMP-CRP\textsuperscript{Mt}-mediated regulation of \textit{whiB1} expression. The identification of a second class II CRP\textsuperscript{Mt-binding site (CRP2) that is occupied when cAMP levels increase and inhibits \textit{whiB1} activation by CRP\textsuperscript{Mt} bound at the class I site (CRP1) indicates that \textit{whiB1} expression \textit{in vivo} should be maximal at intermediate cAMP concentrations. Hence, the following model for cAMP-responsive \textit{whiB1} expression provides the simplest explanation for the data described here. The locations of the two CRP\textsuperscript{Mt}-binding sites (-58.5 and -37.5) are such (centers of the sites are separated by 21 bp) that they are located on the same face of the DNA helix. At low concentrations of the CRP\textsuperscript{Mt}-cAMP complex, CRP1 is preferentially occupied (Fig. 3A); the \(\alpha\)-CTD of RNAP docks downstream of CRP1 (Fig. 5B), and transcription of \textit{whiB1} is activated via a class I mechanism (Fig. 8). Activation of class I promoters by \textit{E. coli} CRP occurs solely by recruiting RNAP to the promoter by increasing the binding constant for the formation of the RNAP-promoter complex (reviewed in Ref. 10). However, the permanganate footprints suggest that low (nonrepressing; Fig. 4A) concentrations of CRP\textsuperscript{Mt} or CRP\textsuperscript{Mt}-cAMP do not significantly enhance open complex formation at the \textit{whiB1} promoter compared with reactions lacking CRP\textsuperscript{Mt} (Fig. 5A) indicating that a step after open complex formation is at. Further experimental work will be needed to identify the mechanism by which this is achieved.

At higher CRP\textsuperscript{Mt} or CRP\textsuperscript{Mt}-cAMP concentrations, both CRP1 (class I position) and CRP2 (class II position) are occupied (Fig. 3A). Occupation of both CRP1 and CRP2 sites would
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Class I

\[ \text{whiB1 low cAMP} \]

\[ \text{CRP1} \]

\[ -58.5 \]

\[ \text{AR1} \]

\[ +1 \]

Class II

\[ \text{whiB1} \]

\[ \text{CRP2} \]

\[ -37.5 \]

\[ \text{AR2} \]

\[ +1 \]

Class III

\[ \text{whiB1 high cAMP} \]

\[ \text{CRP3} \]

\[ -12.5 \]

\[ \text{AR3} \]

\[ +1 \]

FIGURE 8. Architecture of CRP-dependent promoters. The diagram shows the arrangement of nucleoprotein complexes formed at typical class I, class II, and class III CRP-dependent promoters. At class I promoters, the center of the CRP dimer (shown in ribbon form) is positioned at \(-61.5, -71.5, -81.5, \) or \(-91.5\) upstream of the transcript start, placing it on the same face of the DNA helix (horizontal line) as RNAP (shown as unfilled ellipses). This arrangement allows the C-terminal domain of the RNAP \(\alpha\)-subunit (\(\alpha\)-CTD) to interact directly with activating region 1 (AR1) of the downstream subunit of the CRP dimer (\(\bullet\)). At class II promoters, the CRP dimer is centered at or close to \(-41.5\) and is again on the same face of the DNA helix as RNAP. At these promoters multiple interactions between CRP and RNAP are possible, with contacts between AR1 of the upstream subunit of the CRP dimer and \(\alpha\)-CTD, and between activating region 2 (AR2) of the downstream subunit of the CRP dimer and the N-terminal domain of the RNAP \(\alpha\)-subunit (\(\alpha\)-NTD; \(\mathbb{H}\)), and activating region 3 (AR3) of the same CRP subunit and the RNAP \(\beta\)-factor (\(\star\)). Class III promoters have tandem CRP sites in class I and class II locations allowing AR1, AR2, and AR3 contacts with RNAP. For \(E.\ coli\) CRP, these protein-protein interactions recruit RNAP to CRP-dependent promoters (10). For \(M.\ tuberculosis\) Pwbi1 at low cAMP-CRPMt concentrations, CRP1 is occupied and expression is activated, not by RNAP recruitment but by enhancing a step after open complex formation, i.e., promoter clearance. At high cAMP-CRPMt concentrations, CRP1 and CRP2 are occupied. This arrangement has some similarities with the class III architecture, but because the CRP1 and CRP2 sites are immediately adjacent, there is insufficient space to accommodate the \(\alpha\)-CTD between the tandem CRP dimers resulting in inhibition of transcription by preventing \(\alpha\)-CTD from docking with DNA thereby inhibiting productive interaction of RNAP with Pwbi1 (indicated by the double-headed arrow).

leave little space between the two CRPMt dimers, preventing the formation of a typical class III nucleoprotein complex and thus the RNAP \(\alpha\)-CTD is displaced, which is likely to result in either poor or unproductive binding of RNAP to the \(\text{whiB1}\) promoter (Fig. 8). In this way, occupation of CRP2 by CRPMt antagonizes activation by CRPMt bound at CRP1 resulting in inhibition of \(\text{whiB1}\) transcription. Because the concentration of cAMP in \(M.\ tuberculosis\) increases during infection of macrophages (5, 7), this suggests that \(\text{whiB1}\) might be expressed transiently during infection. Although the available microarray datasets (50, 51) do not suggest that \(\text{whiB1}\) expression responds transiently at the time points sampled, this study indicates that the possibility that \(\text{whiB1}\) is transiently expressed during infection should be tested by obtaining high resolution long time course gene expression data to determine the significance of any such transient expression for \(M.\ tuberculosis\) pathogenesis.

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