A TetR Family Transcriptional Factor Directly Regulates the Expression of a 3-Methyladenine DNA Glycosylase and Physically Interacts with the Enzyme to Stimulate Its Base Excision Activity in Mycobacterium bovis BCG*

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Background: No transcriptional factor has been found to directly regulate expression of the DNA glycosylase in bacteria to date.

Results: BCG0878c negatively regulates the expression of MbAAG and stimulates its base excision activity in Mycobacterium bovis BCG.

Conclusion: BCG0878c regulates the function of MbAAG both at the transcriptional and post-translational levels.

Significance: Our findings reveal a novel mode of regulation of 3MeA DNA glycosylase.

3-Methyladenine DNA glycosylase recognizes and excises a wide range of damaged bases and thus plays a critical role in base excision repair. However, knowledge on the regulation of DNA glycosylase in prokaryotes and eukaryotes is limited. In this study, we successfully characterized a TetR family transcriptional factor from Mycobacterium bovis bacillus Calmette-Guerin (BCG), namely BCG0878c, which directly regulates the expression of 3-methyladenine DNA glycosylase (designated as MbAAG) and influences the base excision activity of this glycosylase at the post-translational level. Using electrophoretic mobility shift assay and DNase I footprinting experiments, we identified two conserved motifs within the upstream region of mbaag specifically recognized by BCG0878c. Significant down-regulation of mbaag was observed in BCG0878c-overexpressed M. bovis BCG strains. By contrast, about 12-fold up-regulation of mbaag expression was found in bcg0878c-deleted mutant M. bovis BCG strains. β-Galactosidase activity assays also confirmed these results. Thus, BCG0878c can function as a negative regulator of mbaag expression. In addition, the regulator was shown to physically interact with MbAAG to enhance the ability of the glycosylase to bind damaged DNA. Interaction between the two proteins was further found to facilitate AAG-catalyzed removal of hypoxanthine from DNA. These results indicate that a TetR family protein can dually regulate the function of 3-methyladenine DNA glycosylase in M. bovis BCG both at the transcriptional and post-translational levels. These findings enhance our understanding of the expression and regulation of AAG in mycobacteria.

A timely repair of DNA damage derived from various exogenous and endogenous factors is important in all organisms for maintaining genomic stability. Several DNA repair systems have evolved to correct such DNA lesions, of which base excision repair is one of the most widely studied (1–3). Excision of various alkylated nucleobases is catalyzed by DNA glycosylases, which recognize and excise the damaged base via hydrolysis of N-glycosidic bonds, leaving a basic site (AP-site) that is subsequently excised by an AP endonuclease (2, 4).

Most 3-methyladenine (3MeA)2 DNA glycosylases, including 3MeA, 3-methylguanine (3MeG), 7-methylguanine (7MeG), eA, hypoxanthine (Hx), and 3,N2-ethenoguanine (eG), recognize and excise a wide range of damaged bases resulting from alkylation and deamination (5). Many organisms have more than one type of 3MeA DNA glycosylase. However, mammalian cells have only one kind of 3MeA DNA glycosylase, namely, AAG/MPG (the term AAG is used hereafter), which has broad substrate specificity (6).

AAG was initially thought to exist only in mammalian cells. However, genome sequencing has revealed different homologs of aag in Bacillus subtilis, Borrelia burgdorferi, and several mycobacterial species, such as Mycobacterium tuberculosis, Mycobacterium bovis, and Mycobacterium smegmatis (7). As the causative agent of tuberculosis, M. tuberculosis is a leading cause of death worldwide (8). M. tuberculosis is an intracellular pathogen and its success is linked to its ability to adapt to the environment of the host macrophages, where they are exposed to various potential DNA-damaging assaults, including host-generated antimicrobial reactive oxygen intermediates and reactive nitrogen intermediates (9–11). The conservation of aag genes in multiple mycobacterial species suggests that it may be involved in the regulation of bacterial survival within host macrophages during infection. The activities of AAGs in M.

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2 The abbreviations used are: 3MeA, 3-methyladenine; 3MeG, 3-methylguanine; BCG, bacillus Calmette-Guerin; Hx, hypoxanthine; MbAAG, M. bovis 3-methyladenine DNA glycosylases; NTA, nitrilotriacetic acid.
**TetR Protein Regulates the Function of AAG**

*TB* and *M. smegmatis* have been clearly characterized (12). However, regulation of the DNA glycosylase remains poorly understood, and no transcriptional factor has been successfully characterized as a direct regulator of expression of the enzyme in mycobacterial or any other bacterial species.

*M. bovis* bacillus Calmette-Guerin (BCG) is a live vaccine that has been an important weapon for preventing TB worldwide for over 7 decades (13). BCG is an attenuated strain and possesses high genome identity with the genome of pathogenic *M. tuberculosis* (7, 13). Therefore, *M. bovis* BCG has been widely used as a model strain for studying gene regulation and pathogen-host interaction of *M. tuberculosis*. In this study, we characterized a TetR family transcriptional factor in *M. bovis* BCG named BCG0878c (13) that directly regulates the expression of 3-methyladenine DNA glycosylase, which is encoded by BCG1726 (mbag). Furthermore, we show that the regulator affects base excision activity of the DNA glycosylase by protein-protein interaction. These findings provide the first evidence for regulation of AAG activity in mycobacterial species.

**EXPERIMENTAL PROCEDURES**

**Plasmid, Strains, Enzymes, and Reagents—*Escherichia coli* BL21 strains and pET28a plasmid were purchased from Novagen and used to express mycobacterial proteins. The pGEX plasmid was purchased from Pharmacia. Ni²⁺-nitritolactate-agarose was obtained from Qiagen and used to purify His-tagged proteins. Enzymes, including restriction enzymes, ligase, dNTPs, and DNA polymerase, and all antibiotics were purchased from Takara. Reagents and strains for bacterial one-hybrid assays and two-hybrid assays were purchased from Stratagene. Polymerase chain reaction primers and oligonucleotides were synthesized by Invitrogen.

**Cloning, Expression, and Purification of Recombination Proteins—**The genes *mbag* and *bcg0878c* were amplified using specific primers from the *M. bovis* BCG genome and cloned into the pET28a or pGEX expression vector. Expression and purification of recombinant proteins were accomplished according to the procedures described previously (14). Purified proteins were >95% pure as determined by SDS-PAGE and subsequent staining by Coomassie Blue. Protein concentrations were quantitated by spectrophotometric absorbance at 260 nm according to Gill and von Hippel (15).

**Electrophoretic Mobility Shift Assay—**Long DNA fragments with more than 50 base pairs were amplified using specific primers from the *M. bovis* BCG genomic DNA and cloned into the pET28a or pGEX expression vector. Expression and purification of recombinant proteins were accomplished according to the procedures described previously (14). Purified proteins were >95% pure as determined by SDS-PAGE and subsequent staining by Coomassie Blue. Protein concentrations were quantitated by spectrophotometric absorbance at 260 nm according to Gill and von Hippel (15).

**Bacterial One-hybrid Assay—**A bacterial one-hybrid reporter system, which has been described previously (17), was used to examine interactions between the transcriptional factor and promoter. The promoter was cloned into pBXcmT, which is a derivative of the bacterial two-hybrid pBT bait plasmid (Invitrogen), whereas the transcription factor was cloned into pTRG (Invitrogen). A co-transformant containing pBX-R2031/pTRG-R3133 plasmid served as the positive control and a co-transformant containing the empty vectors pBX and pTRG served as the negative control. The selective medium plate, on which the positive co-transformants were expected to grow well, contained 20 mM 3-amino-1,2,4-triazole, 16 µg/ml of streptomycin, 15 µg/ml of tetracycline, 34 µg/ml of chloramphenicol, and 50 µg/ml of kanamycin. All co-transformants were spotted onto the screening medium and grown at 37°C for 3 to 4 days, then photographed.

**Chromatin Immunoprecipitation—**Exponentially growing cells (1 liter) of *M. bovis* BCG containing the recombinant vector pMV261-embg0878c were fixed with 1% formaldehyde for 20 min. The fixation was then stopped with 125 mM glycine and kept static for 5 min. Cross-linked cells were harvested and resuspended in 10 ml of TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 0.1% Triton X-100). Cells in suspension were broken up using an ultrasonic disruptor (Scientz) 4 times at 300 W for 5 min each. The average DNA fragment size was determined to be ~0.5 kb. About 1 ml of the supernatant was taken out and a 100-µl sample was saved as the input fraction, whereas the remaining 900 µl of the supernatant was incubated with 10 µl of BCG0878c rabbit antiserum (or preimmune serum) for 3 h at 4°C. The complexes were immunoprecipitated with 50 µl of 50% Protein A-Sepharose for 1 h. The immunocomplex was recovered by centrifugation, and cross-linking was reversed for 6 h at 65°C. The DNA samples of the input and ChIP were purified and analyzed by PCR as described previously (18).

**Quantitative Real-time PCR (RT-PCR)—**RNA was extracted from wild-type and recombinant *M. bovis* BCG strains (BCG/pMV261, bbg0878c::hyg, and BCG/pMV261-bgg0878c) as described previously (19). After digestion of DNA by I (Fermentas) to eliminate genomic DNA contamination, gene-specific primers were used to synthesize first-strand cDNAs using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer’s instructions. Every PCR (20 µl) contained 10 µl of 2× SYBR Green Master Mix Reagent (Applied Biosystems), 1 µl of cDNA samples, and 1 µl of gene-specific primers. Reactions were performed in a Bio-Rad CFX RT-PCR machine, as described previously. Amplification specificity was assessed by conducting a melting curve analysis. Expression levels of different genes were normalized to the levels of sigA transcripts (20). Fold-change in gene expression was calculated by the 2ΔΔCt method (21). Average relative expression levels and standard deviations were determined from three independent experiments.

**Analysis of β-Galactosidase Activity—**β-Galactosidase activity experiments were performed in *M. smegmatis*. A series of operon-lacZ fusion plasmids derived from pMV261 (22) was constructed. The recombinant plasmids were then transformed into *M. smegmatis* to obtain corresponding reporter
strains. All strains were grown in 7H9 at 37 °C to an A660 of 1.0. Small aliquots were plated onto 7H10-Kan-Xgal solid medium, and the remaining cells were harvested to measure β-galactosidase activity as described previously (23).

**DNase I Footprinting Assay**—The 152-bp fragment containing the *bcg0878c* promoter region was amplified with specific primers labeled with FITC. The amplified products were purified with BioFlux PCR DNA purification kit (BioFlux) and then subjected to the same binding reaction as in EMSA. DNase I footprinting was performed as described previously (24). Every reaction mixture was treated at 25 °C for 3 min with DNase I (1 unit, Fermentas). The samples were phenol-extracted, ethanol-precipitated, and eluted in 15 µl of distilled water, then added to the HiDi Formamide and GeneScan-500LIZ size standard. Final fragments were analyzed with an Applied Biosystems 3730XL DNA analyzer (manufactured by Tsingke Company, Wuhan). Electropherograms were analyzed and aligned using the GENEMAPPER software (Applied Biosystems).

**Co-immunoprecipitation Assays**—Co-immunoprecipitation was used to examine interactions between BCGB0878c and MbAAG in *M. bovis* BCG, according to the procedure described previously with some modifications (25). Exponentially growing cells (1 liter) of *M. bovis* BCG containing the recombinant vector pMV261-BCG0878c were harvested, resuspended, and lysed in 10 ml of buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). About 1 ml of the supernatant was incubated with 4 µl of MbAAG mouse antiserum (or preimmune serum as a negative control) for 4 h at 4 °C. About 25 µl of Protein A-Sepharose and 2 units of DNase I (Fermentas) were then added to the reaction, and the mixture was incubated for another hour. The immune complex was collected and washed with the same buffer. A Western blot assay was conducted at last to detect BCGB0878c using a rabbit anti-BCG0878 antibody.

**Surface Plasmon Resonance Analysis**—The interaction between BCGB0878c and MbAAG was analyzed on a BIAcore 3000 instrument (GE Healthcare) according to previously published procedures (26). The assays were performed at 25 °C. His-tagged BCGB0878c proteins were immobilized onto NTA (nitrilotriacetic acid) chips. The purified GST-MbAAG protein was diluted in running buffer (100 mM HEPES-NaOH, pH 7.5, 50 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl) and passed over the immobilized NTA chips. For negative controls, GST proteins, GST-MbDnaA (26), GST-MbUdgB (27, 28), and heat-denatured GST-MbAAG were substituted for GST-MbAAG. Each analysis was performed in triplicate. Several overlay plots were produced to depict the interactions using BIAevaluation 3.1 software.

The interaction between the replication origin DNA (OriC) and MbDnaA was also assayed by surface plasmin resonance using biotinylated OriC immobilized onto streptavidin chips (GE Healthcare). Each analysis was performed in triplicate. Plots were produced using data obtained when different combinations of DnaA alone or together with BCGB0878c were passed over the chip.

**Construction of bcg0878c, a Deletion Mutant of M. bovis BCG, and Southern Blot Analysis**—A pMind-derived (29) suicide plasmid carrying a hygromycin resistance gene was constructed, and a sacB-lacZ gene were inserted as a selection marker. Knock-out of the *bcg0878c* gene in *M. bovis* BCG (13) was performed as described previously (18). Southern blot analysis was performed as described previously (19) to confirm the deletion of *bcg0878c*. The probe consisted of a 231-bp fragment of the upstream region of the *bcg0878c* gene, which was amplified using specific primers.

**Assays for DNA Glycosylase Cleavage of Hypoxanthine—**DNA glycosylase assays were carried out according to procedures published previously (30). Briefly, assays were conducted with 5'-labeled FITC duplex DNA containing hypoxanthine at position 13 (5'-CGATAGCATCCT[hypoxanthine]CTCTCTCTCCAT-3'), which was synthesized by Invitrogen. About 2 pmol of such duplexes were incubated with various amount of MbAAG in the reaction buffer (20 mM Tris, 0.1 M KCl, 2 mM EDTA, and 1 mM DTT) at total reaction volumes of 10 µl for 1 h at 37 °C. For Mb UdgB, the assays were carried out in UDG buffer (50 mM Tris-HCl, pH 8.0, 1 mM Na2EDTA, 1 mM DTT, 25 µg ml−1 of BSA) (27, 28). The reactions were stopped by adding 1.2 µl of 1 M NaOH and heating at 70 °C for 30 min. About 11 µl of formamide dye was then added to this mixture. The products were subjected to electrophoresis on a 20% denaturing urea polyacrylamide gel using 1× TBE buffer at 200 V for 3 h at room temperature. Images were then acquired using a Typhoon scanner (GE Healthcare).

**RESULTS**

The BCGB0878c Protein Recognizes the Upstream Sequence of the *mbaag* Gene—Regulation of the expression of the 3MeA DNA glycosylase gene and its effect on mycobacterial growth remains to be elucidated. Using a bacterial one-hybrid technique (17), we performed a screen to isolate potential regulators of *mbaag* gene expression. A TetR family protein, BCGB0878c, was found to specifically bind with the upstream sequence of *mbaag* gene and *bcg0878c* itself. As shown in Fig. 1, when the reporter plasmid containing either the upstream sequence of the *mbaag* gene (pBX-mbaagp) or *bcg0878c* promoter (pBX-bcg0878cp) was co-transformed with the bait plasmid containing the BCGB0878c regulator (pTRG-bcg0878c) into the *E. coli* reporter strain, the co-transformants grew very well on the screening medium. Positive co-transformants with pTRG-Rv3133c/pBX-Rv2031p, selected based on a previously reported interaction (17), also grew well on the medium. By contrast, no growth was observed for their corresponding self-activated controls or the reported negative control composed of co-transformants with pTRG-Rv3133cΔC/pBX-Rv2031p (19). In addition, no growth was observed in co-transformants containing an unrelated regulator (BCGB3080) and an unrelated promoter sequence (*bcg0002p*). These results suggest that BCGB0878c can bind with the upstream sequence of the *mbaag* gene and *bcg0878c* itself.

Subsequent EMSA further confirmed these specific interactions. As shown in Fig. 1B, clear shifts in bands were observed when either the *bcg0878c* promoter (lanes 1–6) or the *mbaag* promoter (lanes 7–10) was co-incubated with increasing amounts of the BCGB0878c protein. Further competition assays showed that the specific shifted band progressively disappeared when a fixed amount of BCGB0878c protein was mixed together.
with increasing amounts of non-labeled bcg0878c promoter (lanes 12–15). Notably, the unrelated promoter could not competitively inhibit the binding of BCG0878c to the labeled bcg0878c promoter (lanes 16–19). These results indicate that the purified BCG0878c protein can recognize the upstream sequence of the mbaag promoter.

**BCG0878c Specifically Binds to the Upstream Sequence of the mbaag Gene in M. bovis BCG in Vivo**—A bcg0878c-deleted mutant strain of *M. bovis* BCG was generated by gene replacement strategy (Fig. 2, A and B). A Southern blot assay confirmed the deletion of bcg0878c. A signal band of about 1.6 kb was detected using a 231-bp probe from the NarI-digested genomic DNA of the mutant *M. bovis* BCG strain (Fig. 2C). By contrast, a signal band of only about 0.95 kb was detected in the wild-type *M. bovis* BCG strain (Fig. 2C). These results indicate that the bcg0878c gene was successfully deleted from *M. bovis* BCG.

Furthermore, CHIP assays were conducted to examine the binding of BCG0878c to the upstream sequence of the mbaag gene and its own promoter in *M. bovis* BCG. As shown in Fig. 1D, both upstream sequence fragments could be specifically recovered by immunoprecipitation using BCG0878c antiserum, whereas the preimmune serum failed to precipitate significant amounts of DNA. By contrast, the promoter of an unrelated gene, bcg0002p, could not be recovered by BCG0878c antiserum. In the bcg0878c-deleted strain, however, neither the antiserum nor the preimmune serum could precipitate the above mentioned promoter fragments. These results strongly support the idea that the BCG0878c protein can specifically bind to the upstream sequence of the mbaag gene as well as its own promoter.

**Characterization of the DNA-box Motif Recognized by BCG0878c**—Several truncated DNA fragments from the upstream region of the bcg0878c gene, designated as PS1-S4 (Fig. 3A), were used to precisely determine the conserved motifs in the region. As shown in Fig. 3A, a clear DNA-protein complex was observed only for PS2 and PS3, whereas no band was observed for PS1 and PS4. Within the upstream region of the mbaag gene, only two fragments, designated as UP and MID (Fig. 3B), were recognized by the BCG0878c protein. To further determine the binding motif of BCG0878c, the PS3 fragment was labeled with FITC and used for DNase I footprinting assays. As shown in Fig. 4A, the region around 5’-TACTGACAGCT-GCGTAGTCAGAATT-3’ was protected when increasing amounts of the BCG0878c protein were incubated with DNase I. This region extended from −45 to −18 (relative to the translational start codon) in the coding strand (Fig. 4B).

We next examined potential motifs in the protected region by aligning the PS2 fraction with the MID fraction. Two DNA box motifs, namely, AAGbox1 (5’-AACGTGC-3’) and AAGbox2 (5’-AGTCAGAATT-3’), were found within the regions of both the mbaag promoter and the bcg0878c promoter (Fig. 4, B and C). We further confirmed that these two DNA box motifs are essential for the recognition of BCG0878c by designing a series of DNA substrates. As shown in Fig. 5A, BCG0878c proteins are capable of binding with substrates containing AAGbox1 (fotS5), AAGbox2 (fotS3), or both (fotS1), but are incapable of binding with sequences flanking the protected region (fotS5) or substrates containing only mutated motifs (fotS4). These results clearly show that DNA-box motifs AAGbox1 and AAGbox2 are the binding sites recognized by BCG0878c.
BCG0878c Negatively Regulates MbAAG Expression—We next sought to determine the effect of BCG0878c-mediated regulation on the expression of mbaag. Using quantitative real-time PCR assays, we compared the fold-change in mbaag gene expression in both wild-type and bcg0878c-deleted mutant strains. As shown in Fig. 6A, expression of MbAAG was strikingly up-regulated (about 12-fold higher) (p < 0.05) in the BCG0878c-deleted strain compared with wild-type, whereas expression of bcg0002, whose promoter lacks the motifs recognized by BCG0878c, did not change. By contrast, when BCG0878c was overexpressed using a pMV261-derived recombinant plasmid in M. bovis, expression of mbaag was down-regulated about 4-fold (p < 0.05), which was consistent with previous assays in the bcg0878c-deleted strain.

Using β-galactosidase as a reporter gene, we constructed a series of promoter-lacZ reporter plasmids to further confirm the effect of the BCG0878c regulator on the expression of mbaag in M. smegmatis. As shown in Fig. 6B, the promoter hsp60 strongly promoted the expression of lacZ. This strain had high β-galactosidase activity (above 2300 Miller units) and appeared deep blue on 7H10 plates, suggesting that the assay worked well. Expression of lacZ under the mbaag promoter was ~500 Miller units. However, when the BCG0878c regulator was induced together with the mbaag promoter, β-galactosidase activity was 5-fold lower than that observed with mbaag promoter alone (~100 Miller units) (Fig. 6B). By contrast, β-galactosidase activity changed little whether BCG0878c was induced or not when an unrelated promoter (bcg0002p), whose fragment does not contain motifs recognized by BCG0878c, was used in the assay under similar conditions. Taken together, these results indicate that BCG0878c is a negative regulator of MbAAG expression.

BCG0878c Physically Interacts with MbAAG—Our results showed that BCG0878c can directly interact with the upstream sequence of mbaag in M. bovis BCG. Interestingly, a recent screen in M. tuberculosis H37Rv also found that BCG0878c potentially interacts with MbAAG. Notably both of these genes contain almost the same sequence as those in M. bovis BCG. To address this issue, we characterized the interaction between BCG0878c and MbAAG, as well as its effect on the activity of MbAAG.

We first utilized a bacterial two-hybrid system to detect a possible interaction between the BCG0878c protein and MbAAG. As shown in Fig. 7A, co-transformants with pTRG-
bcg0878c/pBT-mbaag grew well on the selective medium, whereas no growth was observed for their self-activated controls or for co-transformants with an unrelated protein, BCG3080. Co-immunoprecipitation assays were further used to confirm the interaction in M. bovis BCG in vivo. Protein A beads capable of conjugating with mouse anti-MbAAG antibody makes it possible to detect BCG0878c associated with MbAAG raised from Protein A. As shown in Fig. 7B, when BCG0878c was expressed directly through a pMV261 recombinant plasmid in M. bovis, BCG0878c could be specifically co-immunoprecipitated with anti-MbAAG antibody from the M. bovis cell extraction, but not from the bcg0878c-deleted strain extract. Western blot assays clearly showed the specific signal for BCG0878c associated with MbAAG in vivo, whereas no obvious signal was detected if anti-MbAAG antibody was absent from the reactions.

Furthermore, surface plasmin resonance assays also confirmed specific interaction between the two purified proteins, BCG0878c and MbAAG. As shown in Fig. 7C, when increasing amounts of MbAAG (0.2–0.8 μM) were passed over the (His)6-BCG0878c immobilized NTA chip, a corresponding increase in response was observed. Strikingly, when 0.8 μM MbAAG was passed over the chip, a response of about 500 response units was observed (Fig. 7C, left panel). No significant response was obtained when heated GST-MbAAG or an unrelated protein, including GST proteins, GST-MbUdgB and GST-DnaA (Fig. 7C, right panel), was immobilized on the chip. Thus, our results show that BCG0878c can physically interact with MbAAG both in vitro and in vivo.

BCG0878c Stimulates the Binding Activity of MbAAG for Damaged DNA—We further measured the effect of BCG0878c on the DNA-binding activity of MbAAG. Before doing this, we tested the binding of MbAAG alone to damaged duplexes containing Hx. We found that MbAAG could bind Hx-containing substrates very well (Fig. 8A, lanes 1–6) compared with normal DNA duplexes (Fig. 8A, lanes 7–11). However, heat-inactivated MbAAG could not bind to the damaged substrates. Furthermore, we examined the effect of BCG0878c on the DNA-binding activity of MbAAG. As shown in Fig. 8B (left panel), addition of increasing amounts of BCG0878c (0.2 to 0.8 μM) led to an increase in MbAAG binding to damaged DNA substrates.
corresponding increase in the amounts of the shifted DNA substrates. By contrast, an unrelated protein, BCG3080 (lanes 7–12), did not have any influence on the effect of MbAAG on DNA binding. Furthermore, neither BCG0878c alone nor BCG3080 alone was able to bind to the damaged substrates (lanes 2 and 8). Therefore, our results indicate that BCG0878c

FIGURE 5. EMSA for DNA-binding activity of BCG0878c on different substrates with or without conserved motif sequences. A, four DNA substrates with or without conserved motif sequences were designed and synthesized. The conserved motif sequence is underlined. B, EMSA for the DNA-binding activity of BCG0878c on different substrates. Each of four oligonucleotide substrates was mixed with 0.1 to 0.4 μM BCG0878c protein. BCG0878c is capable of binding with substrates containing AAGbox1 motif (fotS2), AAGbox2 motif (fotS3), or both of them (fotS1) but not with sequences flanking the protected region (fotS5) or substrates with both motifs mutated (fotS4).

FIGURE 6. Effect of the BCG0878c regulator on the expression of mbaag. A, assays for the expression level of mbaag in wild-type, bgc0878c-deleted, and BCG0878c-overexpression M. bovis BCG strains. RT-PCR assays for the relative expression levels of target genes in bgc0878c-deleted M. bovis strains (left panel) and BCG0878c overexpression strains (right panel) were performed. Expression levels of genes were normalized using sigA as an invariant transcript, and an unrelated gene bgc0002 was used as a negative control. All data were analyzed using the 2^ΔΔCt method. Error bars show the variant range of the data derived from three independent biological replicates. p value of the results calculated by unpaired two-tailed Student’s t test is indicated by an asterisk on top of each column. No RT represents genomic DNA contamination control. B, β-galactosidase activity assays for the effect of BCG0878c on the expression of mbaag. A series of lacZ-fused plasmids were created and are listed in the left panel. Exponentially growing M. smegmatis mc2155 containing various plasmids were plated onto 7H10 plates containing 30 μg/ml of kan and 50 μg/ml of X-Gal and are shown in the middle. β-Galactosidase activity is shown as Miller units in the right panel, and values presented are the average of three independent experiments. Error bars indicate S.D. For statistical analysis, two-way analysis of variance with Bonferroni multiple comparison tests were performed with a p value of ≤0.05, marked by an asterisk, indicating statistical significance.
can physically interact with MbAAG and stimulate its binding to damaged DNA.

BCG0878c Stimulates the Cleavage Activity of MbAAG on Damaged DNA—DNA glycosylases usually initiate base excision repair by binding, then excising aberrant DNA bases. We subsequently performed DNA glycosylase assays and determined the effect of BCG0878c on this process. As shown in Fig. 9A, when increasing concentrations of MbAAG (40 to 160 nM) were added into the reaction, more Hx-containing substrates were cleaved, which led to corresponding increases in the production of 12-mer products (Fig. 9A, left panel). By contrast, MbAAG did not show base excision activity for normal DNA substrates (Fig. 9A, right panel).

Because BCG0878c maintains a close interaction with MbAAG, BCG0878c can likely regulate the activity of MbAAG at the protein level. We further examined the effect of BCG0878c on the base excision activity of MbAAG. As shown in Fig. 9B, when the amount of MbAAG remained constant (90 nM) while increasing concentrations of BCG0878c (0.2 to 1.2 μM) were added into the reaction, a corresponding increase in production of 12-mer products was clearly observed, indicating that BCG0878c can stimulate the cleavage activity of MbAAG. As a negative control, BCG3080 did not have much effect on MbAAG (Fig. 9A, left panel). By contrast, MbAAG did not show base excision activity for normal DNA substrates (Fig. 9A, right panel).

Because BCG0878c maintains a close interaction with MbAAG, BCG0878c can likely regulate the activity of MbAAG at the protein level. We further examined the effect of BCG0878c on the base excision activity of MbAAG. As shown in Fig. 9B, when the amount of MbAAG remained constant (90 nM) while increasing concentrations of BCG0878c (0.2 to 1.2 μM) were added into the reaction, a corresponding increase in production of 12-mer products was clearly observed, indicating that BCG0878c can stimulate the cleavage activity of MbAAG. As a negative control, BCG3080 did not have much effect on MbAAG (Fig. 9B, upper right panel). We also used MbUdgB, another mycobacterial DNA glycosylase that does not interact physically with BCG0878c (Fig. 7C), to determine the specific effect of BCG0878c on the base excision activity of MbAAG. Under similar reaction conditions, BCG0878c did not affect the cleavage activity of MbUdgB (Fig. 9B, lower left panel). Furthermore, BCG0878c alone did not show base excision activity (Fig. 9B, lower right panel). Therefore, our results show that BCG0878c enhanced the binding activity of MbAAG to Hx-containing DNA and its base excision activity.
DISCUSSION

3MeA DNA glycosylases can recognize and excise damaged DNA bases. Therefore, they play important roles in base excision repair. However, the regulation of both their gene expression and cleavage activity remains largely unclear. In the present study, we analyzed a TetR family transcriptional factor named BCG0878c in M. bovis BCG, which directly regulates the expression of mbaag and stimulates the base excision activity of the DNA glycosylase via protein-protein interaction. We show that the transcription factor BCG0878c can regulate mbaag at both transcriptional and post-translational levels. Our findings provide a novel model for the regulation of 3MeA DNA glycosylase in mycobacteria.

Previous studies indicated that increasing human AAG levels in cultured human cells induced an MSI phenotype and increased spontaneous frameshift mutation rates in yeast cells (31). Interestingly, the genomes of several mycobacterial species, including M. tuberculosis and M. bovis BCG, contain a highly conserved eukaryote-like AAG gene (32). However, no transcriptional factor has ever been characterized as a regulator of AAG, and the regulatory mechanisms involved remain unclear. In the current study, we isolated for the first time a TetR family transcriptional factor (BCG0878c) that directly binds to the promoter of the mbaag gene and negatively regulates its expression. Interestingly, BCG0878c proteins recognize two DNA box motifs that do not contain palindrome sequences. This indicates that BCG0878c is largely different from most typical TetR-type regulators that usually recognize a palindrome or an inverted repeat (33). Therefore, although structurally similar to TetR, BCG0878c may have evolved some unique characteristics that enable it to flexibly regulate the expression of AAG and physically interact with the protein.

Most transcription factors regulate the expression of target genes at the transcriptional level by binding target promoters directly or becoming part of a large transcriptional preinitiation complex (34, 35). In the present study, we found that BCG0878c has dual functions, on the one hand, it interacts with promoters of the target gene, whereas on the other, it binds with its cognate protein. This mode of regulation is uncommon but not rare, and can be found in an extensively studied type II toxin and antitoxin system (36) in which the toxin and its cognate antitoxin are in the same operon and usually co-expressed to form a stable toxin-antitoxin complex. Both the complex and anti-toxin can bind with the promoters of the system to repress the expression of the whole system (37, 38). However, the mechanism we reported in the present study is intriguing and novel in that, unlike toxins and antitoxins that are usually co-transcribed and placed adjacent to each other in the genome, BCG0878c acts as a trans-acting factor and remotely regulates mbaag. Notably, toxin-antitoxin complexes usually inhibit the function of toxin, but the BCG0878c protein stimulates the excision activity of Mbaag.

A previous study showed that human AAG can specifically bind with DNA containing one and two base pair loops within...
TetR Protein Regulates the Function of AAG

repetitive sequences that are involved in genomic instability (39). However, little is known about the regulation of the cleavage activity of AAG for damaged DNA substrate. In the current study, besides acting as a transcriptional regulator, BCG0878c was found to physically interact with MbAAG and stimulate its DNA excision activity. Therefore, on the one hand, BCG0878c can inhibit expression of the mbaag gene by targeting the mbaag promoter. On the other hand, BCG0878c can elevate excision activity of the MbAAG protein on damaged DNA substrates to repair damaged nucleotides and ensure bacterial genomic stability. In our model, BCG0878c mainly works as a transcription factor and inhibits the expression of MbAAG to maintain its function at the post-translational level. This kind of cleavage ability of MbAAG on damaged DNA substrates to promote genomic stability. In our model, BCG0878c mainly works as a transcription factor and inhibits the expression of MbAAG to maintain its function at the post-translational level. This kind of cleavage ability of MbAAG on damaged DNA substrates to repair damaged nucleotides and ensure bacterial genomic stability. In our model, BCG0878c mainly works as a transcription factor and inhibits the expression of MbAAG to maintain its function at the post-translational level. This kind of dual function of BCG0878c at transcriptional and post-translational levels may play important roles for the survival of mycobacterial species, such as M. tuberculosis and M. bovis, in host macrophage where they are confronted with severe surroundings and exposed to various potential DNA-damaging assaults (9–11).

In summary, a TetR-type transcription factor BCG0878c was successfully isolated from M. bovis BCG and shown to negatively regulate the expression of MbAAG by directly binding to the mbaag promoter. Furthermore, the regulator can stimulate the cleavage activity of mbaag for damaged DNA substrate by physically interacting with the DNA glycosylase. Thus, we have characterized a protein that not only acts as a transcriptional regulator of the expression of mbaag but also directly modulates the activity of the DNA glycosylase at the post-translational level. These findings provide novel insights into the mechanism of AAG regulation in mycobacteria.

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