

## *Mycobacterium tuberculosis* FurA Autoregulates Its Own Expression

Claudia Sala,<sup>1</sup> Francesca Forti,<sup>1</sup> Elisabetta Di Florio,<sup>1</sup> Fabio Canneva,<sup>2</sup> Anna Milano,<sup>2</sup>  
Giovanna Riccardi,<sup>2</sup> and Daniela Ghisotti<sup>1\*</sup>

Dipartimento di Genetica e di Biologia dei Microrganismi, Università di Milano, Milan,<sup>1</sup> and Dipartimento di Genetica e di Microbiologia A. Buzzati Traverso, Università di Pavia, Pavia,<sup>2</sup> Italy

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**The *furA-katG* region of *Mycobacterium tuberculosis*, encoding a Fur-like protein and the catalase-peroxidase, is highly conserved among mycobacteria. Both genes are induced upon oxidative stress. In this work we analyzed the *M. tuberculosis furA* promoter region. DNA fragments were cloned upstream of the luciferase reporter gene, and promoter activity in *Mycobacterium smegmatis* was measured in both the presence and absence of oxidative stress. The shortest fragment containing an inducible promoter extends 45 bp upstream of *furA*. In this region, –35 and –10 promoter consensus sequences can be identified, as well as a 23-bp AT-rich sequence that is conserved in the nonpathogenic but closely related *M. smegmatis*. *M. tuberculosis* FurA was purified and found to bind upstream of *furA* by gel shift analysis. A ca. 30-bp DNA sequence, centered on the AT-rich region, was essential for FurA binding and protected by FurA in footprinting analysis. Peroxide treatment of FurA abolished DNA binding. Three different AT-rich sequences mutagenized by site-directed mutagenesis were constructed. In each mutant, both *M. tuberculosis* FurA binding *in vitro* and *pfurA* regulation upon oxidative-stress in *M. smegmatis* were abolished. Thus, *pfurA* is an oxidative stress-responsive promoter controlled by the FurA protein.**

Fur-like proteins are widespread in both gram-negative and gram-positive bacteria. Their major role in the regulation of iron uptake systems in response to environmental iron was first described for *Escherichia coli* (for a review, see reference 14). Under iron-replete conditions, the Fur protein, using Fe<sup>2+</sup> as a corepressor, binds to a 19-bp operator sequence located upstream of the Fur-regulated genes (2, 10, 11, 13).

Fur and Fur-like proteins were found to be involved in regulation of functions as varied as the acid shock response (17), detoxification of oxygen radicals (12, 18), production of toxins and virulence factors (20, 31), and metabolic pathways (24, 29). These observations led Fur-like proteins to be considered not only transcriptional repressors but also global regulators.

The *E. coli* Fur protein (17 kDa) contains a nonclassical helix-turn-helix DNA binding domain and is active as a homodimer (7). *In vivo* assays showed that the N-terminal domain is involved in DNA binding and that the C-terminal domain is involved in dimerization (29). Biochemical analysis indicated that repressor activation occurs upon a conformational change due to metal binding to the C-terminal domain (8, 15, 30). The Fur protein contains two metal ion binding sites: the iron binding regulatory site and a tightly binding zinc site that plays a role in stability of the protein (1, 19).

Several redox-sensing proteins of the Fur family have been identified in various bacteria. Their role in the oxidative stress response might be mediated via metal-catalyzed oxidation of the protein or a change in the oxidation state of the bound metal ion (4, 16). This is the case for CatR in *Streptomyces coelicolor*, which regulates its own gene and the catalase CatA

by binding to operator sequences upstream of their promoters under reducing conditions (16). Similarly, in *Streptomyces reticuli*, FurS in the thiol-reduced form specifically binds to a motif upstream of the *furS* gene (25).

The *furA* gene has been identified in *Mycobacterium tuberculosis* as well as other mycobacterial species (6, 23, 26). *S. reticuli* FurS and *M. tuberculosis* FurA show 53% identity, and the predicted secondary structures of the two proteins are similar. Moreover, typical motifs involved in metal binding and several cysteines that may sense the oxidative stress are conserved (25, 32). Thus, the two proteins might play similar roles in responding to oxidative stress.

The presence of an oxidative stress-inducible promoter, immediately upstream of the *furA* gene, has been reported previously for *M. tuberculosis*, *Mycobacterium smegmatis*, and *Mycobacterium bovis* BCG (21, 23, 27). In *M. bovis* BCG, the 5' end of the *furA* transcript was mapped in correspondence to the start codon of the *furA* gene (27). A 23-bp AT-rich sequence, overlapping the –35 region of *pfurA*, was identified and found to be conserved among mycobacteria.

In this study, we define more precisely the sequence of the *pfurA* inducible promoter of *M. tuberculosis* and demonstrate that FurA negatively controls its own transcription by binding to this region in a redox-dependent manner.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *E. coli* XL1-Blue (5) was grown in LD medium (28) supplemented, when necessary, with kanamycin (50 µg/ml). *M. smegmatis* mc<sup>2</sup>155 was grown in LD medium containing 0.2% (vol/vol) glycerol and 0.05% (vol/vol) Tween 80 and supplemented when necessary with kanamycin (20 µg/ml). The complete *M. tuberculosis* genome sequence is reported under GenBank accession no. AL123456.

**Construction of plasmids.** DNA fragments from *M. tuberculosis* were amplified by PCR with specific primers and cloned in pSG10ter (23) upstream of the *luxAB* reporter gene. The sequences of the primers will be provided upon request. All of the inserted fragments were sequenced.

\* Corresponding author. Mailing address: Università degli Studi di Milano, Dipartimento di Genetica e di Biologia dei Microrganismi, Via Celoria 26, 20133 Milan, Italy. Phone: 39-02-50315020. Fax: 39-02-50315044. E-mail: Daniela.Ghisotti@unimi.it.

**Mutagenesis.** Complementary oligonucleotides carrying each of the 6-bp substitutions indicated in Fig. 6A were used in combination with external primers to amplify the *M. tuberculosis* -154/+33 region. The amplified fragments were either end labeled, purified, and used in gel retardation assays (see below) or cloned in pSG10ter upstream of *luxAB*.

**Luciferase assay.** Independent cultures of *M. smegmatis* mc<sup>2</sup>155 carrying the indicated plasmids were grown in LD medium at 37°C, the turbidity of the cultures at 600 nm was adjusted to 0.03, and the cultures were sampled and either mock treated or treated for 1 h at 37°C with 0.062 mM H<sub>2</sub>O<sub>2</sub>. One hundred microliters of 1% (vol/vol) decyl aldehyde solution (Sigma-Aldrich) in ethanol was then added to 1 ml of a 1:10 dilution of the culture, and luminescence was measured over 10 s, using a Stratec luminometer.

**Cloning and purification of FurA.** The *M. tuberculosis furA* gene was amplified from cosmid DNA. The primers used contained restriction sites for *Bam*HI and *Eco*RI. The PCR fragment was digested with the indicated restriction enzymes and cloned into the pGEX6P1 expression vector (Pharmacia). Recombinant plasmids were transformed into *E. coli* XL1-Blue, and colonies were isolated on Luria-Bertani plates containing 100 µg of ampicillin per ml. Clones were tested for the presence of the correctly inserted *furA* gene and sequenced.

Recombinant protein expression was obtained by growing cells for 16 h at 26°C in LB medium supplemented with ampicillin (100 µg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside) (50 µM). The culture was then harvested by centrifugation and stored at -20°C. The cell pellet from 250 ml of culture was resuspended in 5 ml of 1× phosphate-buffered saline (8 g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub> per liter, 0.24 g of KH<sub>2</sub>PO<sub>4</sub> per liter) and sonicated on ice. The lysate was centrifuged for 30 min at 13,000 rpm, and the supernatant fraction was applied to 0.5 ml of GS4B resin (Pharmacia) that was previously equilibrated with 10 volumes of 1× phosphate-buffered saline. All of the subsequent purification steps were performed according to manufacturer's instructions. Protein elution was done after digestion with PreScission protease (Pharmacia) for 16 h at 4°C; both the glutathione *S*-transferase (GST) tag and protease remain tightly bound to the chromatographic matrix, allowing their separation from the protein.

The protein concentration was evaluated by the Bradford assay with bovine serum albumin as a standard.

**Gel retardation assay.** DNA probes for gel shift experiments were amplified by PCR with specific primers containing a *Bam*HI site at one 5' end, digested with *Bam*HI, labeled with the Klenow enzyme and [ $\alpha$ -<sup>32</sup>P]dATP, and purified from polyacrylamide gels. Binding reaction mixtures in 10 µl of binding buffer [20 mM Tris HCl (pH 8), 1 mM dithiothreitol (DTT), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.05 mg of poly(dI-dC) per ml, 0.05 mg of bovine serum albumin per ml, 10% glycerol, and 200 µM NiSO<sub>4</sub>] containing about 7.4 fmol of the probe (40,000 cpm) were incubated with purified FurA protein (12 µM) for 20 min at room temperature. Reaction mixtures were loaded on an 8% polyacrylamide gel containing 40 mM Tris acetate (pH 8) and 200 µM NiSO<sub>4</sub>. Gels were run at 120 V at 4°C, dried, and exposed to PhosphorImager (Molecular Dynamics) detection.

**DNase I footprinting.** The pMYT101 plasmid was digested with either *Bam*HI or *Eco*RI, labeled with the Klenow enzyme and [ $\alpha$ -<sup>32</sup>P]dATP, and digested with *Eco*RI or *Bam*HI, respectively. The probes were extracted from a 5% polyacrylamide gel and eluted overnight in 10 mM Tris HCl (pH 7.4). Binding reactions were performed as described for the gel retardation assay, with 60,000 cpm of the probe in a final volume of 100 µl. DNase I digestion was carried out by treatment with 1 µl of DNase I (1.5 ng/µl) and 1 mM CaCl<sub>2</sub> for 1 min at room temperature. Reactions were stopped by addition of 50 µl of Stop buffer (0.1 M EDTA, 0.8% sodium dodecyl sulfate [SDS], 1.6 M NH<sub>4</sub>-acetate, 300 mg of herring sperm DNA per ml). DNA was precipitated with 350 µl of ethanol, dried, and resuspended in 7 M urea-20 mM Tris HCl (pH 8) with tracking dyes. Samples were loaded on a 7 M urea-6% polyacrylamide sequencing gel. Maxam-Gilbert A+G sequencing reactions were performed as described previously (22).

## RESULTS

**Mapping of an oxidative stress-inducible promoter upstream of *furA* in *M. tuberculosis*.** We previously reported the presence of an oxidative stress-inducible promoter upstream of the *furA* genes in both *M. smegmatis* and *M. tuberculosis* (23). We mapped the *M. tuberculosis* promoter by cloning several DNA fragments upstream of the reporter *luxAB* gene and measuring luciferase activity expressed in *M. smegmatis* mc<sup>2</sup>155 (Fig. 1).

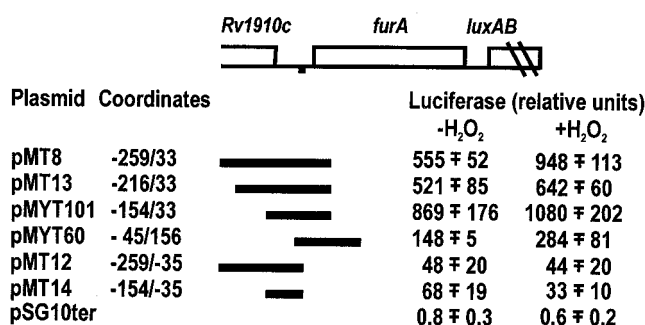


FIG. 1. Oxidative stress induction of luciferase by plasmids carrying *M. tuberculosis* DNA regions. (Top) Schematic representation of the *M. tuberculosis furA* region. The AT-rich sequence upstream of *furA* is indicated by a black square. The DNA fragments cloned in pSG10ter are indicated, and the coordinates of the fragments are reported. The *M. tuberculosis* coordinates are arbitrary, with coordinate +1 corresponding to the first nucleotide of the *furA* gene. (Bottom) Luciferase activity expressed in *M. smegmatis* mc<sup>2</sup>155 transformed by the plasmids was measured, as described in Materials and Methods, both in the absence of (-H<sub>2</sub>O<sub>2</sub>) and after (+H<sub>2</sub>O<sub>2</sub>) oxidative stress. The mean values ± standard deviations for two to four independent clones tested are reported.

Luciferase activity was measured both before and after peroxide treatment of the cells, as described in Materials and Methods, and the results are reported in Fig. 1. Plasmids pMT8, pMT13, and pMYT101 expressed high luciferase activity that increased upon oxidative stress. In contrast, pMT12 and pMT14 did not express luciferase. The latter plasmids lack the -35/+33 region that appears to be essential for promoter activity. Deletion of the region upstream of position -45 (pMYT101) did not abolish luciferase-inducible expression, although the activity was reduced to about 20 to 25%, suggesting that the region upstream of position -45 contributes to promoter efficiency.

Upstream of the *furA* gene, -35 and -10 promoter consensus sequences and a 23-bp AT-rich region overlapping the -35 sequence were observed (Fig. 2A). This latter sequence is highly conserved in *M. bovis*, *M. smegmatis*, and *Mycobacterium fortuitum* upstream of *furA* (Fig. 2B), thus indicating that a regulatory protein might bind to this region.

It was found previously that FurS of *S. reticuli* is able to bind to a region that shares some homology with the mycobacterial 23-bp AT-rich sequence (25) (Fig. 2B). This suggested that mycobacterial FurA might bind upstream of its own promoter. Thus, we decided to express and purify *M. tuberculosis* FurA and test the ability of the protein to bind this DNA region.

**Purification of *M. tuberculosis* FurA.** The *M. tuberculosis furA* gene was amplified by PCR and cloned in pGEX6P1, creating a fusion with the GST protein (see Materials and Methods). Expression of the GST-FurA fusion protein was induced with IPTG in *E. coli* XL1-Blue cultures, and the protein was recovered by using glutathione-Sepharose. After cleavage with PreScission, the partially purified FurA protein was separated by SDS-polyacrylamide gel electrophoresis (Fig. 3A). One major and three minor bands were visualized, which might represent different conformations of the protein. The major band has an apparent molecular mass of about 20 kDa, as expected for FurA.

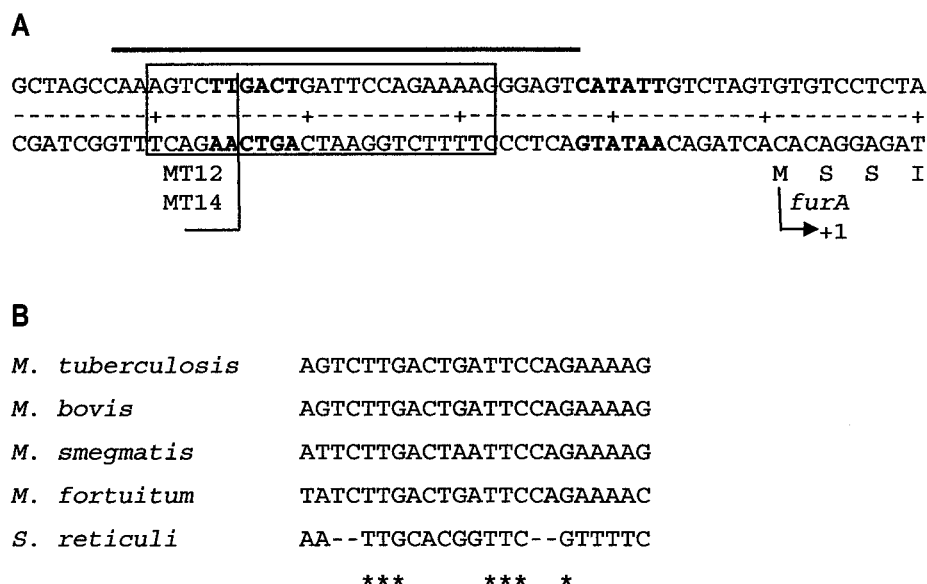


FIG. 2. Sequence of the *M. tuberculosis* DNA fragment containing the oxidative stress-inducible promoter. (A) Sequence of the -50/+10 *M. tuberculosis* region. The coordinates are arbitrary, with coordinate +1 corresponding to the first nucleotide of the *furA* gene. The -35 and -10 sequences are in boldface. The 23-bp AT-rich region is boxed. The ends of the fragments cloned in MT12 and MT14 are indicated. The region protected by FurA is indicated by a line above the sequence. (B) Alignment of the AT-rich DNA sequences upstream of *furA* in *M. tuberculosis*, *M. bovis*, *M. smegmatis*, and *M. fortuitum* and the sequence upstream of *furS* in *S. reticuli*. The conserved bases are indicated by asterisks.

Reducing treatment by addition of 1 or 5 mM DTT changed the electrophoretic mobilities of all FurA bands (Fig. 3A). In contrast, addition of peroxide did not alter the electrophoretic pattern. This indicates that the purified protein is in a (partially) oxidized state. Moreover, the change in electrophoretic mobility suggests that FurA might be a redox-sensing protein.

**Binding of FurA to the region upstream of *M. tuberculosis furA*.** The ability of FurA to bind to the region upstream of the *furA* gene was tested by gel shift experiments. A preliminary test, using a DNA fragment that covers the -154/+33 *M. tuberculosis* region, indicated that FurA was able to bind under reducing conditions (1 mM DTT), whereas peroxide treatment prevented FurA binding (Fig. 3B). A change in FurA protein conformation upon oxidative treatment was likely to abolish DNA binding. Gel shift analysis was thus performed with the addition of 1 mM DTT to the binding reaction mixture.

In order to map the binding region, several DNA fragments overlapping the 5' region of *M. tuberculosis furA* were amplified by PCR, end labeled, and used in gel shift experiments with the purified FurA protein. The fragments tested and the results of the gel shift assay with FurA are reported in Fig. 4. Three fragments (fragments 902, 1105, and 1109) were shifted in the presence of FurA protein (Fig. 4B). All of them contain the 23-bp AT-rich region identified upstream of *furA*. On the other hand, two fragments lacking this sequence (fragments 1107 and 1111) were not shifted in the presence of FurA. Addition of 3,000 M specific unlabeled DNA efficiently competed FurA binding to both the 1105 and 1109 fragments, whereas no effect was observed upon addition of a similar amount of unspecific DNA (Fig. 4C). Thus, we concluded that a specific region located between positions -50 and -6 was sufficient for FurA binding.

**Identification of the FurA binding site in *M. tuberculosis*.** Footprinting analysis was performed in order to identify the

FurA binding site upstream of *furA*. The 187-bp *EcoRI-BamHI* *M. tuberculosis* region carried by MYT101 was digested, end labeled at position +33 and used in footprinting experiments in the presence of increasing concentrations of FurA and 1 mM DTT (Fig. 5A). A weakly protected region extending from

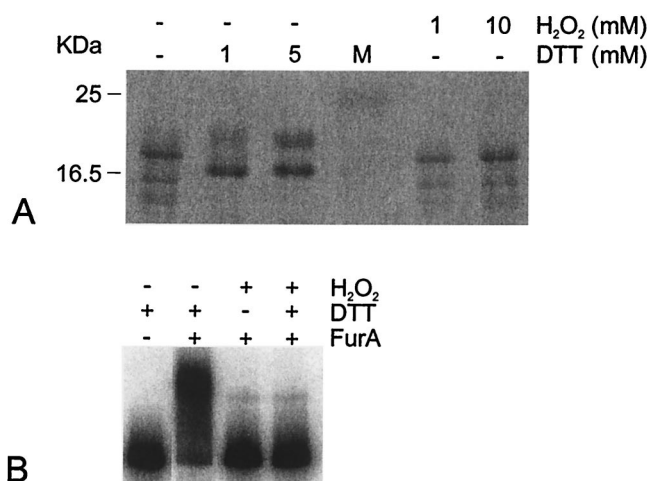


FIG. 3. Electrophoretic mobility of the FurA protein and binding of FurA to DNA under different redox conditions. (A) Purified *M. tuberculosis* FurA (1.7 µg) expressed in *E. coli* was run in the first lane of an SDS-15% polyacrylamide gel. Equivalent samples were mixed with either DTT or H<sub>2</sub>O<sub>2</sub> at the concentrations indicated above the lanes before being loaded on the gel. Lane M, molecular mass markers, with sizes indicated on the left. (B) Gel shift caused by FurA. The DNA probe covers the -154/+33 *M. tuberculosis* region. FurA (10 µM) was added, as indicated above the lanes, to <sup>32</sup>P-labeled DNA probe in the presence of 200 µM Ni<sup>2+</sup>, and complexes were resolved on a Tris-acetate-8% polyacrylamide gel containing 200 µM Ni<sup>2+</sup>. DTT (1 mM) and H<sub>2</sub>O<sub>2</sub> (10 mM) were added to the incubation buffer as indicated.

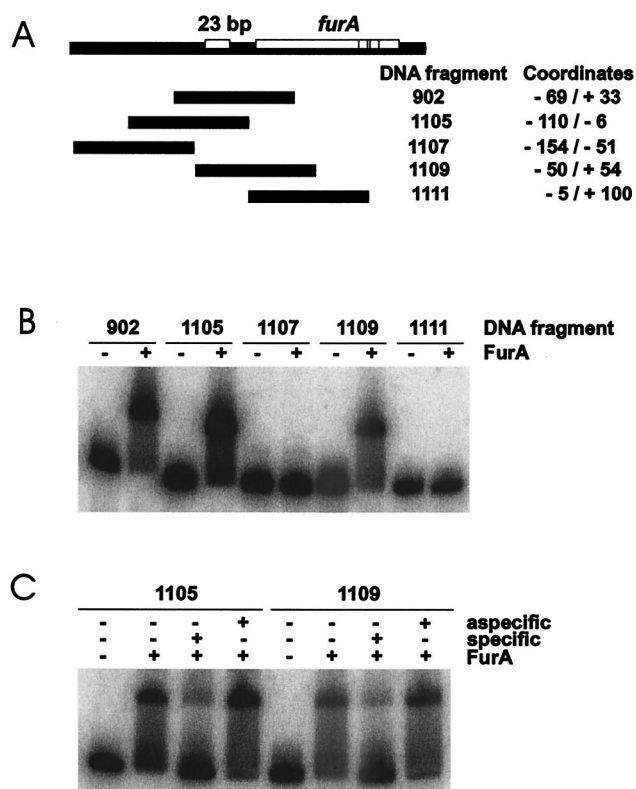


FIG. 4. FurA binds DNA fragments covering the *M. tuberculosis furA* upstream region. (A) Map of the DNA fragments. (B) Gel shift caused by FurA. FurA binding was performed as described in the legend to Fig. 3, except that 12  $\mu$ M FurA was added. (C) Specificity of FurA binding. Specific (same DNA fragment) and nonspecific (1111 DNA fragment) competitor DNA was added to each binding reaction mixture at 3,000 M.

position  $-14$  to  $-43$  and centered on the AT-rich region was identified (Fig. 2A). The protected region overlaps the  $-35$  promoter sequence, thus suggesting that FurA binding might control transcription of the *furA* gene. The same DNA fragment was labeled at the opposite end (position  $-154$ ), and footprinting was repeated (Fig. 5B). The presence of the  $-14/-43$  protected region, immediately upstream of *furA*, was confirmed. Moreover, at high FurA protein concentrations we observed an extension of the protection that suggests cooperative binding of FurA proteins on the DNA.

**Mutagenesis of the FurA operator sequence.** Direct mutagenesis of the *furA* operator sequence was performed by PCR amplification with mutagenized primers (see Materials and Methods). Three different mutants were obtained, each differing from the wild-type sequence for a 6-bp stretch within the AT-rich sequence. The mutations are reported in Fig. 6A. Gel shift analysis with FurA indicated that each mutation almost completely prevented FurA binding (Fig. 6B), thus suggesting that the mutant sequence is directly involved in FurA operator recognition.

In order to test the effect of the mutations in the operator sequence on promoter activity and inducibility by oxidative stress, we cloned the fragments upstream of the luciferase gene and measured luciferase activity in *M. smegmatis* mc<sup>2</sup>155 (Table 1). pMYT103 and pMYT104 expressed high luciferase

activity (about 9,500,000 relative light units [RLU]), compared to about 10 times less in the wild-type pMYT101. Both pMYT103 and pMYT104 were not induced upon oxidative stress. Thus, the promoter in the mutants appears to be fully functional, whereas repression by FurA was lost.

Expression of luciferase activity by pMYT102, in which the  $-35$  sequence has been mutated, was about 1,600,000 RLU. In this case, the mutation likely affected promoter activity. However, also in this case, no induction upon oxidative stress could be observed.

All three mutagenized promoters, in which FurA binding was abolished, were no longer controlled upon oxidative stress.

## DISCUSSION

In *M. tuberculosis*, the region encompassing the  $-45/+33$  sequence relative to the *furA* gene start codon was found to be sufficient to promote transcription and to respond to oxidative stress induction. In this region of *M. bovis* BCG, the 5' end of *pfurA* and canonical  $-35$  and  $-10$  promoter sequences have been previously identified (23). Moreover, *M. tuberculosis* DNA fragments covering this region were found to express a downstream reporter gene (21, 23, 27). Extension of the cloned

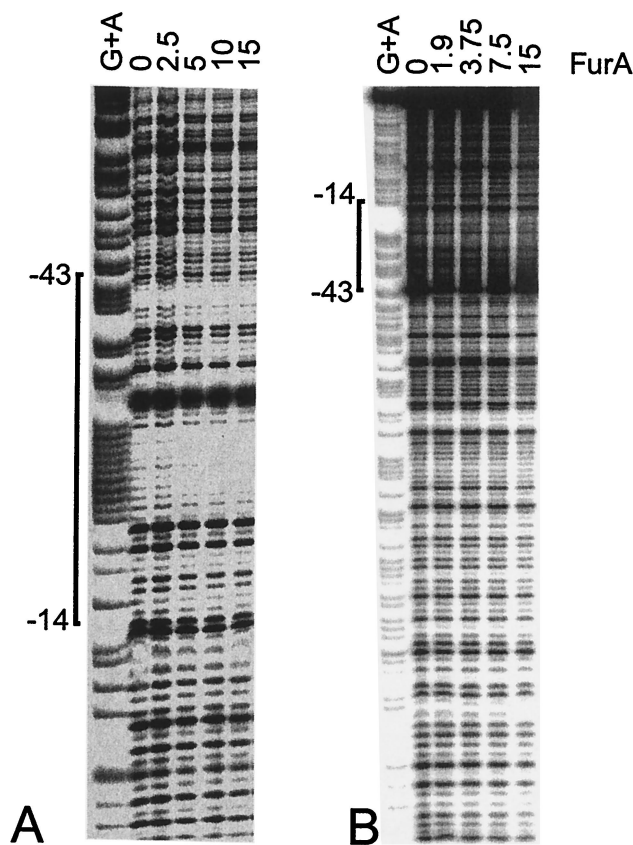


FIG. 5. Footprint analysis of the FurA binding site upstream of *M. tuberculosis furA*. The purified  $-154/+33$  DNA region, labeled at either the  $+33$  end (A) or the  $-154$  end (B), was incubated in the presence of the amount of FurA protein (in micrograms) indicated above the lanes for 20 min at room temperature and digested with DNase I as described in Materials and Methods. Maxam-Gilbert A+G sequences of the same fragments were loaded in the first lanes.

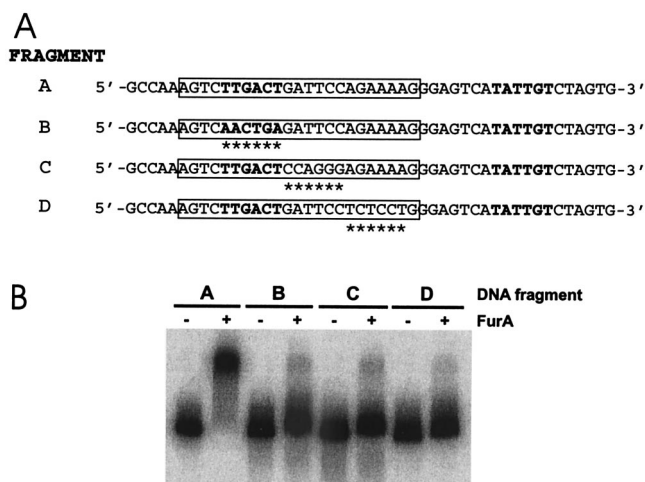


FIG. 6. Effect of mutations in the *furA* operator sequence on FurA binding. (A) Sequences of the mutations. Fragment A, wild type. Fragment B, C, and D carry different 6-bp substitutions within the AT-rich region (boxed). The mutant bases are indicated by asterisks. The -10 and -35 sequences are in boldface. (B) Gel shift of the mutant fragments caused by FurA. FurA binding was performed as described in the legend to Fig. 4.

region upstream of position -45 increased promoter efficiency about fourfold, suggesting the presence of sequences that might be involved in regulation of transcription from *pfurA*.

In this work, we demonstrate that *pfurA* is negatively controlled by the mycobacterial FurA protein, which binds upstream of the *furA* gene. Thus, FurA autoregulates its own expression.

Band shift experiments mapped the Fur box in the 50-bp region upstream of the *furA* gene. Binding appears to be specific, since it was not competed by addition of a 3,000 M concentration of nonspecific DNA. By footprinting analysis a protected region covering the -14/-43 sequence upstream of the initiation codon was identified. This box overlaps the -35 consensus sequence of the *pfurA* promoter, suggesting that FurA competes with RNA polymerase for binding to the operator-promoter region. Moreover, the Fur box is centered on a 23-bp AT-rich sequence, which is highly conserved in mycobacteria and in *S. reticuli* (Fig. 2B). In *S. reticuli* FurS was found to bind to this sequence, thus repressing transcription of the downstream operon. Addition of metal ions enlarged the protected region, whereas oxidative conditions abolished binding (25). It might be hypothesized that FurA binding occurs by a similar mechanism. In our experiments, Ni<sup>2+</sup> ions were always present in the binding buffer. In their absence binding appeared to be weak or absent (data not shown). Moreover, reducing conditions were necessary, since in the presence of peroxide FurA binding did not occur.

A gel shift of the DNA fragments was observed only in the presence of FurA at a concentration higher than 3 μM (data not shown), with a molar ratio of FurA to DNA of about 4 × 10<sup>-4</sup>. The large amount of protein required to observe the band shift may suggest that most of our protein preparation was inactive. Since different FurA isoforms were observed in SDS-polyacrylamide gel electrophoresis, we hypothesize that only one of them may be active.

It should be noted that in our footprinting experiments protection by FurA was not complete, since most of the bands were reduced but still visible. Only a slight increase of the protection could be observed by increasing the FurA concentration. This might indicate that not all of the DNA molecules were bound by FurA. Whether this is a consequence of an internal equilibrium or of the in vitro conditions used was not established. However, the band shift experiments are in good agreement with the protected region identified by footprinting analysis, and this result was confirmed by footprinting on the opposite DNA strand.

At high FurA concentrations, we observed an enlargement of the protected region, which extended to most of the DNA fragment used. Thus, FurA binds to the Fur box, and from this nucleoprotein complex several FurA molecules may be added cooperatively, covering the DNA fragment. A similar mechanism was proposed for Fur autoregulation in *Helicobacter pylori* (9).

The 23-bp AT-rich region is unique in the *M. tuberculosis* genome, since no homologous sequences could be found by the FINDPATTERNS program of the Genetics Computer Group package unless at least five mismatches were allowed. This might indicate either that *M. tuberculosis* FurA is not a global transcription regulator or that different DNA regions are recognized and bound by FurA. The 23-bp AT-rich region is almost completely conserved in *M. smegmatis*, and our data indicate that *M. smegmatis* FurA recognizes and binds to the *M. tuberculosis* sequence cloned upstream of the reporter gene. Thus, FurA autoregulation appears to be common to both mycobacterial species.

The addition of DTT changes FurA electrophoretic mobility, indicating that the protein senses the redox state. This property is likely to have a central role in the oxidative stress response: *furA* transcription is repressed by FurA binding, and, upon oxidative stress, the oxidized FurA protein loses its DNA affinity and transcription from *pfurA* can take place.

Three different mutations of a 6-bp stretch within the AT-rich sequence prevented FurA binding, thus confirming that the sequence is specifically recognized by the FurA protein. The effect of the mutations was to eliminate the control of *furA* expression. It is interesting that all of the mutations introduced in *M. smegmatis* caused a complete loss of the response to oxidative stress. Even more interesting is the fact that two mutations (pMYT103 and pMYT104) exhibited a very high

TABLE 1. Luciferase activity expressed by mutant DNA fragments.

Plasmid <sup>a</sup>	Luciferase RLU (10 <sup>3</sup> ) <sup>b</sup>	
	Without H <sub>2</sub> O <sub>2</sub>	With H <sub>2</sub> O <sub>2</sub>
pMYT101	869 ± 176	1,080 ± 202
pMYT102	1,682 ± 370	1,638 ± 332
pMYT103	9,528 ± 1,054	8,549 ± 1,314
pMYT104	9,632 ± 2,566	9,616 ± 2,844

<sup>a</sup> The plasmids were derived from pSG10ter by insertion of the *M. tuberculosis* -154/+33 fragments upstream of the *luxAB* reporter gene. pMYT101 carries the wild-type sequence, and pMYT102, pMYT103, and pMYT104 carry the B, C, and D mutant sequences indicated in Fig. 6, respectively.

<sup>b</sup> Luciferase activity was measured as described in the legend to Fig. 1. The cultures were treated with 0.062 mM H<sub>2</sub>O<sub>2</sub> for 1 h. The mean values ± standard deviations for four independent clones tested are reported.

luciferase activity when cloned upstream of the reporter *luxAB*, i.e., about 10 times higher than wild-type activity. Apparently, both 6-bp substitutions did not alter any sequence essential for promoter activity. This suggests that *pfurA* is a very strong promoter and that it is maintained in an almost completely repressed state by FurA itself. Upon oxidative stress several FurA proteins detach from the DNA, and *pfurA* is partially activated. This result also indicates that both *M. smegmatis* and *M. tuberculosis* FurA, which share high homology, are able to interact with *pfurA* promoter sequences.

The third mutant (pMYT102) carries a 6-bp substitution in the  $-35$  sequence. This mutation had a double effect: it completely abolished FurA binding, thus preventing the oxidative stress response, and reduced, but did not abolish, *pfurA* activity. In this case the mutation altered a sequence relevant for promoter activity. Indeed, the presence of an intact  $-10$  sequence enabled RNA polymerase to initiate transcription, although at a reduced rate. Promoters that lack a canonical  $-35$  sequence but are still functional have been reported for *M. tuberculosis* (3).

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