Transcriptional Regulation of *furA* and *katG* upon Oxidative Stress in *Mycobacterium smegmatis*

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The DNA region upstream of *katG* in *Mycobacterium smegmatis* was cloned and sequenced. The *furA* gene, highly homologous to *Mycobacterium tuberculosis furA*, mapped in this region. The *furA-katG* organization appears to be conserved among several mycobacteria. The transcription pattern of *furA* and *katG* in *M. smegmatis* upon oxidative stress was analyzed by Northern blotting and primer extension. Although transcription of both *furA* and *katG* was induced upon oxidative stress, transcripts covering both genes could not be identified either by Northern blotting or by reverse transcriptase PCR. Specific transcripts and 5′ ends were identified for *furA* and *katG*, respectively. By cloning *M. smegmatis* and *M. tuberculosis* DNA regions upstream of a reporter gene, we demonstrated the presence of two promoters, *pfurA*, located immediately upstream of the *furA* gene, and *pkatG*, located within the terminal part of the *furA* coding sequence. Transcription from *pfurA* was induced upon oxidative stress. A 23-bp sequence overlapping the *pfurA* −35 region is highly conserved among mycobacteria and streptomycetes and might be involved in controlling *pfurA* activity. Transcription from a cloned *pkatG*, lacking the upstream *pfurA* region, was not induced upon oxidative stress, suggesting a *cis*-acting regulatory role of this region.

Despite the seriousness of tuberculosis in the world, efforts in prevention and control of this disease suffer from the lack of detailed knowledge of the mechanisms used by pathogenic mycobacteria for survival within host cell macrophages. A variety of mechanisms contribute to the survival of *Mycobacterium tuberculosis* in the host, and protection against oxidative stress is one of the primary defenses (1). However, little is known about regulation and expression of the genes involved in oxidative stress resistance. Most bacteria, exposed to toxic oxygen metabolites, exhibit an adaptive response, expressing several genes involved in detoxification of oxygen species (4). The pathogenic mycobacteria have a limited, nonprotective oxidative stress response, which in the case of *M. tuberculosis* is restricted to induction of a single gene, *katG*, encoding a catalase-peroxidase (14, 28). Consistently, the KatG protein is an important virulence factor in *M. tuberculosis*, since its expression is required for growth and persistence in mouse and guinea pig model systems (17). The importance of a better understanding of the elements involved in *katG* regulation is increased by its implication in the innate susceptibility and acquired resistance to the front-line antituberculosis drug isoniazid (isonicotinic acid hydrazide) (9, 33). Indeed, inactivation of *katG* is frequently found among isoniazid-resistant *M. tuberculosis* strains (32).

Response to oxidative stress in mycobacteria differs from the OxyR-dependent induction observed in gram-negative bacteria (25). Indeed, both *M. tuberculosis* and *Mycobacterium smegma-* *tis* lack a functional *oxyR* gene (6, 8, 31). In all the mycobacterial species studied, the DNA region immediately upstream of the *katG* genes is highly conserved and encodes the *furA* gene, suggesting a putative involvement of the FurA protein in *katG* regulation. Recently, Zahrt et al. (31) demonstrated that in *M. smegmatis* a knockout *furA* mutant overexpressed the catalase-peroxidase KatG and that this altered phenotype is complemented by expression of FurA; thus, FurA appears to be a negative regulator of *katG*.

Fur-like proteins are transcriptional repressors that exhibit a Fe^{2+}-dependent DNA binding activity and regulate several genes involved in iron metabolism (10). There is an intimate relationship between iron metabolism and oxidative stress: indeed, the cytotoxic effects of reactive oxygen species are largely mediated by iron, and it has been shown elsewhere that regulators of *Escherichia coli* involved in oxidative stress response, OxyR and SoxRS, activate the expression of Fur, the global repressor of ferric iron uptake (34). *E. coli* Δ*fur* mutants are more sensitive to hydrogen peroxide than are Fur-proficient strains (30). In *M. smegmatis*, hydrogen peroxide sensitivity is increased by iron starvation (18); in *Staphylococcus aureus*, Fur is necessary for oxidative stress resistance (15); and in *Streptomyces* spp., Fur-like proteins regulate catalase-peroxidase genes in an iron-dependent manner (13, 35).

In this work, using *M. smegmatis* as a model system, we investigated transcription of *furA* and *katG* after oxidative stress. Specific transcripts and promoters were identified for *furA* and *katG*, respectively. Although transcription of both genes was induced upon oxidative stress, an inducible promoter could be identified only upstream of *furA*. A similar transcriptional regulation was found in the *furA-katG* region of *M. tuberculosis*.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* XL1-Blue (3) was grown in LD medium (26), supplemented when necessary with kanamycin (50 μg/ml). *M. smegmatis* mc²155 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) or spectinomycin (100 μg/ml).

Cloning and sequencing of *M. smegmatis furA* gene. A cosmid library of *M. smegmatis* mc²155 was produced by standard procedures in the vector Tropist4 (7). The library was screened using a 380-bp DNA internal to the *katG* coding region, obtained by PCR amplification with a couple of primers (218 and 219 [see Fig. 1]) based on the published sequence (GenBank accession no. U46844).

Construction of plasmids. The pSG10ter vector was derived from pSG10 (11) by inserting the *E. coli* Rho-independent Ω terminator (24) upstream of the reporter gene *luxAB*, in order to reduce basal luciferase expression. Plasmid pSM128 is an integrative vector carrying the *lacZ* reporter gene (29). Plasmid pSG10ter and pSM128 derivatives, containing DNA fragments from either *M. smegmatis* (MS3, MYS42, MYS44, MYS48, MYS65, and MYS66) or *M. tuberculosis* (MT3, MYT43, and MYT45), were obtained by PCR amplification of the regions (the oligonucleotide sequences will be provided upon request). All the inserted fragments were sequenced.

RNA extraction, Northern blotting, and primer extension analysis. Fifteen milliliters of *M. smegmatis* mc²155 cultures (optical density at 600 nm = 0.8), untreated or treated with H_2O_2 for 1 h at 37°C at the concentrations indicated in Fig. 1, was pelleted and resuspended in 100 μl of Tris-EDTA buffer; 75 μl of RNA lysis buffer (4 M guanidinium thiocyanate, 0.01 M Tris [pH 7.5], 0.97% β-mercaptoethanol) was added; and the suspension was sonicated (20 s at 40 W). The RNA was then purified using the SV Total RNA Isolation System (Promega). Twenty micrograms of each RNA sample was fractionated, blotted, and hybridized as described previously (5). The ^{32}P-labeled riboprobes *furA* and *katG* were prepared by SP6 polymerase transcription of pGEMT-easy (Promega) derivatives, carrying the *M. smegmatis* 349–509 (oligonucleotides 240 and 256) and 724-1113 (oligonucleotides 218 and 219) DNA regions, respectively (see Fig. 1). The oligonucleotide probes (indicated in Fig. 1) were 5′ end labeled with T4 polynucleotide kinase as described by Sambrook et al. (27). Potential transcription start sites in the *M. smegmatis furA-katG* region were identified by primer extension analysis (27), using the oligonucleotides indicated.

Reverse transcriptase PCR (RT-PCR). Two micrograms of RNA extracted from *M. smegmatis* mc²155 mock treated and treated for 1 h with either 0.6 or 20 mM H_2O_2 was retrotranscribed with Moloney murine leukemia virus reverse transcriptase (Promega). Samples (50 ng) were amplified with oligonucleotides 254 and 219 and, as a control, with oligonucleotides 218 and 219 and oligonucleotides 240 and 271. The presence of an amplified DNA was evaluated both by ethidium bromide staining and by Southern blot hybridization.

Luciferase assay. Independent cultures of *M. smegmatis* mc²155 carrying the plasmids indicated in Table 1 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 with H_2O_2 (final concentration, 0.125 mM) for 1 h at 37°C. 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. At least three independent clones of each construct were tested.

β-Galactosidase assay. The cultures were grown as described for the luciferase assay to an optical density at 600 nm of 1.7 and mock treated or treated for 1 h with 0.5 mM H_2O_2 at 37°C, and the cells were disrupted by sonication. β-Galactosidase activity of the extracts was measured as described in reference 20. The enzyme activity was expressed as nanomoles of *o*-nitrophenol-β-D-galactopyranoside converted to *o*-nitrophenol minute^{-1} milligram of protein^{-1}.

Nucleotide sequence accession number. The sequence upstream of *katG* was determined and deposited in the GenBank database under accession number AF196484.

RESULTS

Cloning and sequencing of *M. smegmatis furA*. The *katG* region of *M. smegmatis* was identified by screening a cosmid library with an internal *katG* PCR fragment as a probe (see Materials and Methods). Sequence inspection of this region revealed the presence of an open reading frame predicted to encode a polypeptide of 153 amino acids, with a calculated molecular mass of 17 kDa, homologous to Fur-like proteins. The gene was named *furA*, by analogy with other *fur* genes

located upstream of *katG* in mycobacteria (23) (Fig. 1A). A Shine-Dalgarno sequence is present upstream of *katG*, while no typical Shine-Dalgarno sequence was identified upstream of *furA*.

Comparison of the *M. smegmatis* and *M. tuberculosis furA-katG* nucleotide regions by FASTA indicated a 70 to 80% identity in the region starting about 40 bp upstream of the start codon of *furA* and ending near the end of the *katG* coding sequence; the adjacent regions diverge completely. Analysis of the sequence upstream of *furA* indicated the presence of the terminal 149 codons of an open reading frame, encoding a polypeptide highly homologous (129 of 149 amino acids identical) to an *M. tuberculosis* hypothetical oxidoreductase (Gen-Bank accession no. B70697). However, the corresponding gene in *M. tuberculosis* maps in a different part of the genome.

Transcription of *furA-katG* in *M. smegmatis* upon oxidative stress. Transcription of *katG* and *furA* in *M. smegmatis* in response to oxidative stress was analyzed by Northern blotting, and the transcripts were mapped by hybridization with different probes. The RNAs were extracted from mc²155 cultures after 1 h of treatment with different concentrations of hydrogen peroxide (Fig. 1B, C, D, and E). The *katG* coding sequence is 2,223 nucleotides (nt) long. A single transcript, about 2.3 kb long, hybridized with the *katG* riboprobe (Fig. 1B). The same transcript could be observed with the 244 probe, internal to *katG*, and with the 553 probe, encompassing the 3′ end of the *furA* coding sequence (Fig. 1D). With the *furA* riboprobe and with the 554 oligonucleotide, both mapping in the 5′ half of the *furA* coding sequence, the 2.3-kb transcript was not detected, even after prolonged exposure of the filter (Fig. 1C and D). Thus, the *katG* transcript extended to the intergenic region between *furA* and *katG* but did not cover the whole *furA* gene. Immediately downstream of *katG*, a potential Rho-independent termination site was identified by the TERMINATOR program of the GCG package (data not shown).

The basal level of the *katG* mRNA was relatively abundant, and the intensity of the signal varied upon oxidative stress: as determined by PhosphorImager analysis, a two- to threefold increase compared to untreated control was detected upon exposure at low doses (0.1 to 0.6 mM H_2O_2 for 1 h); at higher doses (1 to 10 mM H_2O_2), a decrease was observed that reduced the intensity of the signal to 1.5-fold the basal level. A strong increase (about 10-fold) was present after severe treatment with hydrogen peroxide (10 to 20 mM; the concentration of H_2O_2 causing this induction varied by a factor of 2 in different experiments). The induction at low doses is likely to be the adaptive response, whereas the latter increase might be a response to a global alteration of cell metabolism.

The *furA* riboprobe and the 554 oligonucleotide hybridized to transcripts less than 0.6 kb long, which were better resolved in a polyacrylamide gel (Fig. 1C, D, and E). Although the intensity of these signals was lower than that of *katG* RNA (a three-times-longer exposure was required for their detection), the induction pattern upon oxidative stress appeared similar: a 2.2-fold increase at low doses, followed by a decrease to the basal level at 1 to 5 mM and an approximately 10-fold increase at ≥10 mM H_2O_2 (Fig. 1E). In this latter condition, four RNAs, 390, 430, 480, and 560 nt long, were identified.

Transcripts covering both *furA* and *katG* could not be identified by Northern blotting, not even after prolonged exposures
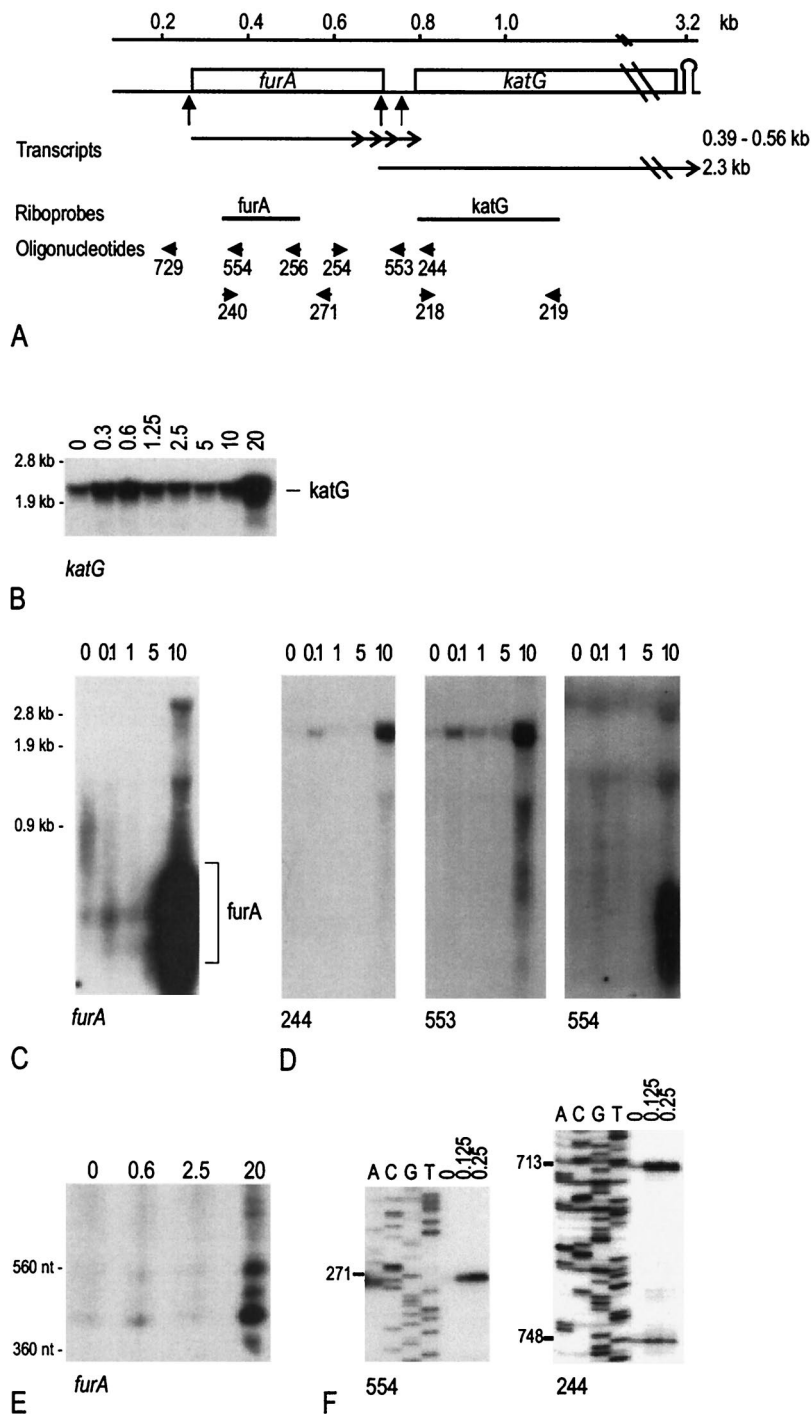
FIG. 1. Transcription of the *furA-katG* region in *M. smegmatis*. (A) Map of the region. The *furA-katG* map of *M. smegmatis* is drawn to scale according to the sequence in the GenBank database with accession no. AF196484 (coordinate 1 corresponds to nt 245 of the deposited sequence). The genes are indicated by boxes. The potential Rho-independent termination site, downstream of *katG*, is shown. The transcripts are indicated by arrows, and the 5′ ends are indicated by vertical arrows below the map. The positions of the riboprobes and oligonucleotides used for Northern blotting, primer extension analysis, and RT-PCR are reported. (B to E) Northern blotting analysis of *furA-katG* transcription upon oxidative stress. RNAs were extracted from a culture of *M. smegmatis* mc²155 treated for 1 h with the millimolar concentration of hydrogen peroxide indicated on the top of the lanes, and equal amounts of each sample (20 µg) were separated by agarose (B, C, and D) or polyacrylamide (E) gel electrophoresis, blotted, and hybridized to the different probes. (B) Riboprobe *katG* (coordinates 724 to 1113); (C and E) riboprobe *furA* (coordinates 332 to 511); (D) the same filter used in panel C was hybridized successively to oligonucleotides 554 (coordinates 366 to 385), 553 (coordinates 715 to 742), and 244 (coordinates 803 to 822). Molecular size markers, run in the same gel, are indicated on the left. For panels C and D, probe 554 was exposed about three times longer. In agarose gels, two nonspecific bands (about 1.5 and 3 kb) were observed after prolonged exposure of the filters (particularly visible in panels C and D, oligonucleotide 554). These bands are likely to be *furA-katG* RNA entrapped by rRNA (5). (F) Primer extension of 5′ ends of *furA* and *katG* transcripts. The primer extension experiments were performed with the 554 (*furA*) and 244 (*katG*) oligonucleotides. Sequence reactions, performed with the same oligonucleotides on MS3 DNA (Table 1), are reported in the first four lanes of the two panels. The coordinates of the 5′ ends are reported on the left of each panel.

TABLE 1. Expression of luciferase and β-galactosidase from *M. smegmatis* and *M. tuberculosis* DNA fragments

Plasmid[a]	Coordinates of mycobacterial fragment[b]	Luciferase (relative units)[c]			β-Galactosidase (relative units)[c]		
		−H$_2$O$_2$	+H$_2$O$_2$	Relative increment	−H$_2$O$_2$	+H$_2$O$_2$	Relative increment
pSG10ter		1.5 ± 0.6	1.9 ± 0.8	0.95 ± 0.04	NT[d]	NT	NT
pSM128*		NT	NT	NT	1.2 ± 0.3	1.3 ± 0.3	1.11 ± 0.02
MS3	137–821	430 ± 127	1,782 ± 297	4.26 ± 0.76	NT	NT	NT
MYS42/MYS65*	222–821	68 ± 8	77 ± 20	1.58 ± 0.06	172 ± 15	241 ± 5.2	1.32 ± 0.005
MYS44/MYS66*	258–821	51 ± 7	35 ± 0.1	0.70 ± 0.09	15.4 ± 0.7	15.3 ± 1.5	0.99 ± 0.045
MYS48	669–821	17 ± 8	13 ± 5	0.78 ± 0.10	NT	NT	NT
MT3	157–766°	106 ± 12	308 ± 36	2.91 ± 0.01	NT	NT	NT
MYT43	264–766°	81 ± 62	97 ± 65	1.40 ± 0.30	NT	NT	NT
MYT45	296–766°	137 ± 33	92 ± 38	0.63 ± 0.12	NT	NT	NT

[a] pSG10ter derivatives; pSM128 derivatives are indicated by an asterisk.

[b] Coordinates of the *M. smegmatis* sequence are arbitrary, with coordinate 1 corresponding to position 245 in the sequence deposited in the GenBank database (accession no. AF196484). Coordinates of the *M. tuberculosis* sequence (°) are arbitrary, with coordinate 1 corresponding to position 2,156,900 in the reverse strand of the *M. tuberculosis* complete genome sequence (GenBank accession no. AL123456).

[c] Luciferase and β-galactosidase activities expressed by *M. smegmatis* mc^{2}155 transformed with the plasmids were measured, as described in Materials and Methods, both in the absence of (−H$_2$O$_2$) and after (+H$_2$O$_2$) oxidative stress. Treatment with 0.125 mM and 0.5 mM H$_2$O$_2$ for 1 h was used in luciferase and β-galactosidase assays, respectively, depending on the different cell concentrations used in the two assays. The $\bar{x}$ values ± standard deviations of three to five independent clones tested are reported. The relative increment of activity upon oxidative stress is also indicated.

[d] NT, not tested.

of the filters. The absence of *furA-katG* transcripts was confirmed by RT-PCR using RNA extracted from both noninduced and H$_2$O$_2$-induced cells (data not shown; see Materials and Methods).

Identification of promoter regions upstream of *katG* and *furA*. The 5′ ends of the transcripts were mapped by primer extension. Using an oligonucleotide internal to the *furA* coding sequence (oligonucleotide 554 in Fig. 1A), a unique 5′ end was identified at nt 271 (Fig. 1F), 1 base upstream of the translation start codon of *furA*. The intensity of the signal increased 9- to 12-fold upon oxidative stress. No other signals could be identified further upstream, neither with this oligonucleotide nor with an oligonucleotide located upstream of *furA* (oligonucleotide 729 in Fig. 1A; data not shown).

With an oligonucleotide internal to the *katG* coding sequence (oligonucleotide 244 in Fig. 1A), two bands were identified at 713 and 748 nt (Fig. 1F). The signal at nt 713 is located within the terminal part of the *furA* coding sequence; the nt 748 end maps in the intergenic region (Fig. 1A). No signals could be identified further upstream; in particular, no signal at nt 271 was observed (data not shown). The position of the two 5′ ends is consistent with the Northern blotting data, which indicated that the 2.3-kb transcript started upstream of *katG*. The nt 713 band increased about fourfold upon oxidative stress, whereas the nt 748 band showed only a less-than-twofold intensification.

In order to identify promoter regions, we cloned fragments of the *M. smegmatis furA-katG* DNA in pSG10ter upstream of the bacterial *luxAB* reporter gene and measured luciferase expressed by the constructs. The cloned fragments, obtained by PCR amplification, are indicated in Table 1. The constructs were tested for luciferase activity in *M. smegmatis* mc^{2}155 both in the absence of and upon oxidative stress. Consistent with the results obtained by Northern blotting, MS3 and MYS42, whose cloned fragments extend, respectively, from nt −135 and nt −50 upstream of *furA* to the first codons of *katG*, expressed a high level of luciferase that was increased upon peroxide treatment. (In MYS42 the increase was about three times lower than that in MS3.)

In contrast, luciferase expression by MYS44, whose DNA region extends from nt −14 upstream of *furA* to the first codons of *katG*, did not increase upon oxidative stress (the slight reduction of luciferase activity observed upon 1 h of H$_2$O$_2$ treatment was probably caused by cell lethality). Moreover, the DNA fragment cloned in MYS48, which covers the terminal part of *furA*, also expressed a low noninducible luciferase activity. These results suggest the following.

(i) MS3 and MYS42 contain an inducible promoter, *pfurA*, which requires the region upstream of −50 for oxidative stress activation. Sequence inspection of this region revealed the presence of potential −35 (TTGACT) and −10 (TAGCCT) consensus sequences. *pfurA* might be responsible for the 5′ end at nt 271 identified by primer extension.

(ii) In the DNA fragment cloned in MYS44, the −35 region of *pfurA* is deleted. The noninducible activity expressed by MYS44 and MYS48 might be due to a weak promoter, *pkatG*, located in the terminal region of *furA*. This promoter might be responsible for the 5′ ends at nt 713 and 748 identified by primer extension. However, transcription from *pkatG* was not induced upon oxidative stress in either MYS44 or MYS48.

To confirm these results, we cloned the same DNA fragments in a different reporter system, using the integrative vector pSM128 and *lacZ* as a reporter gene (Table 1). β-Galactosidase activity expressed by the constructs clearly indicated the presence of two promoters, *pfurA* and *pkatG*, and confirmed that *pkatG* was not induced upon oxidative stress in the absence of the region upstream of −50.

Promoters of *furA* and *katG* in *M. tuberculosis*. Fragments of *M. tuberculosis* DNA of the *furA-katG* region were cloned in pSG10ter. *M. smegmatis* mc^{2}155 was transformed with the plasmids, and luciferase activity was determined (Table 1). The DNA regions cloned were homologous to the corresponding *M. smegmatis* fragments, except for the first 113 nt of MT3. The expression of luciferase activity from the different plasmids

indicated that in *M. tuberculosis furA-katG* transcription is regulated in a way similar to that found in *M. smegmatis*: an inducible *pfurA* promoter is located immediately upstream of *furA*, where −35 (TTGACT) and −10 (TATTGT) sequences were identified. Deletion of the −35 region eliminated inducibility upon oxidative stress (compare MYT43 and MYT45), although a basal level of luciferase activity was still observed.

DISCUSSION

Expression of the *furA-katG* region in *M. smegmatis*. We have cloned and sequenced the *M. smegmatis* region upstream of *katG*. It contains the *furA* gene, which codes for FurA, one of the two Fur-like proteins identified in the *M. tuberculosis* genome. Zahrt et al. (31) have recently sequenced the same region of *M. smegmatis* (GenBank accession no. AF012631). The FurA proteins deduced from the two sequences differ for some residues, with FurA deduced from our sequence presenting a higher similarity to *M. tuberculosis* FurA. The *M. smegmatis* FurA protein conserves both the helix-turn-helix DNA binding domain (35) and the metal binding domain (2), suggesting that this protein might carry out regulatory functions in response to stress conditions such as iron depletion or oxidative stress.

The *furA-katG* organization is conserved in many mycobacteria (23) as well as in the closely related *Streptomyces coelicolor* (13) and *Streptomyces reticuli* (35), and a regulatory role for the FurS protein on expression of catalase-peroxidase has been demonstrated previously in streptomycetes (13, 22). Moreover, it has been demonstrated elsewhere that *katG* expression is enhanced in an *M. smegmatis* Δ*fur* mutant strain, suggesting that FurA negatively regulates *katG* expression (31).

Our transcription analysis in *M. smegmatis* indicates that both *furA* and *katG* are induced upon oxidative stress, although, contrary to what was previously reported for mycobacteria and streptomycetes (13, 19, 22, 23), distinct messengers for each gene were found.

Transcription of *furA*. Four transcripts covering *furA* were detected with a riboprobe encompassing the 5′ half of *furA*. The two longest ones cover the entire *furA* coding sequence, whereas the two shorter terminate before the end of the gene. Thus, they may either represent stable intermediates of the processed *furA* transcript or be produced by premature transcription termination.

The transcription from *pfurA* starts at nt 271, just 1 base upstream of the GTG initiation codon of *furA*. The leaderless mRNA is consistent with the lack of a Shine-Dalgarno sequence upstream of the *furA* gene (12, 16). Upstream of the transcription start point, −35 (TTGACT) and −10 (TAGCCT) consensus sequences are found. Corresponding sequences are present in *M. tuberculosis* upstream of *furA* (TTGACT and TATTGT, respectively). The expression of luciferase and β-galactosidase in the reporter systems used confirmed the presence of a promoter, induced upon oxidative stress, both in *M. smegmatis* and in *M. tuberculosis*. The presence of *pfurA* in *M. tuberculosis* has been recently demonstrated (19).

Deletion of the 222–258 region in *M. smegmatis* and of the equivalent region of *M. tuberculosis* (264–296 [Table 1]) abolished H_2O_2 inducibility. These deletions cover the −35 *pfurA*

sequences. An AT-rich region of 23 bp overlaps the −35 sequence of *pfurA* in *M. smegmatis* (ATTCTTGACTAATTCCAGAAAAG) and in *M. tuberculosis* (AGTCTTGACTGATTCCAGAAAAG). A highly homologous region was found in several mycobacteria upstream of *furA* (*Mycobacterium bovis*, *Mycobacterium fortuitum*, and *Mycobacterium leprae*) and in *S. reticuli* upstream of *furS*, and it has been demonstrated elsewhere that FurS, the protein corresponding to FurA in *Streptomyces*, binds to this region in a redox-dependent manner (22). Similarly, FurA might be a redox-sensing regulator both in *M. smegmatis* and in *M. tuberculosis*. In its reduced form, the mycobacterial FurA protein might bind to the AT-rich region, acting as a repressor of transcription initiation. Upon oxidative stress, the oxidized FurA protein might lose its DNA binding activity, allowing transcription from *pfurA*. Thus, FurA might autoregulate its own expression. Consistently, purified *M. tuberculosis* FurA changed its electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis upon addition of increasing amounts of dithiothreitol (data not shown).

BLAST analysis indicated that the 23-bp AT-rich region is unique in the *M. tuberculosis* genome. Thus, either FurA, unlike other Fur proteins, is not a global regulator or different DNA sequences might be bound by FurA. It appears noteworthy that the high homology between *M. smegmatis* and *M. tuberculosis furA-katG* DNA regions starts exactly at the 23-bp sequence.

Transcription of *katG*. Two possible 5′ ends were identified for the 2.3-kb *katG* transcript. This suggests the presence of two mRNAs, differing by 35 bp in length, that were not resolved by agarose gel electrophoresis. The transcript starting at nt 713 might be partially processed, generating the nt 748 5′ end. Alternatively, transcription may start at both sites. Similarly, for *M. tuberculosis* Mulder et al. (21) identified three potential 5′ ends for *katG* transcripts, two of them internal to the *furA* coding sequence and one in the intergenic region, whereas Master et al. (19) identified a single 5′ end internal to *furA*, 54 nt upstream of the *katG* coding sequence.

The recent work of Master et al. (19), based on S1 nuclease protection experiments, reports that in *M. tuberculosis furA* and *katG* are cotranscribed. Our data, for *M. smegmatis*, support a different hypothesis, with independent transcription of the two genes. It cannot be excluded that transcription in the two mycobacterial species differs. However, S1 protection experiments did not detect a transcript covering both *furA* and *katG*; rather, they indicated the presence of RNAs that cover *furA* and extend within the first few nucleotides of the *katG* coding sequence (the probe used extends to the first 22 nt of *katG*). An *M. tuberculosis* RNA equivalent to the 560-nt-long *furA* transcript that we observed by Northern analysis might be responsible for the 5′ end at *pfurA*.

Upon mild oxidative stress, the 2.3-kb transcript and the intensity of the nt 713 signal increased about three- to fourfold, whereas the nt 748 signal appeared to be less inducible (less than twofold). No consensus promoter sequences were identified upstream of either 5′ signal. However, by cloning this *M. smegmatis* DNA region (MYS44 and MYS66) and the corresponding *M. tuberculosis* DNA region (MYT45) upstream of the luciferase and the β-galactosidase genes, a low promoter activity was detected, which did not increase upon oxidative

stress. Thus, in the reporter system used *pkatG* was not inducible.

The response to oxidative stress of *pkatG* might require the presence of the upstream region, encompassing *pfurA*. Indeed, Master et al. (19) demonstrated that *M. tuberculosis pkatG* was induced upon oxidative stress by using a construct that includes the upstream *furA* region (similar to our MT3 [Table 1]). We suggest that basal *katG* expression depends on the activity of *pkatG* and that *cis*-acting regulatory sites, necessary for activation by oxidative stress, are located in the upstream region. In agreement with the idea that this region contains a promoter activator, Mulder et al. (21) identified in *M. tuberculosis* an upstream activation region required for *pkatG* expression. The upstream activation region, which includes *pfurA*, enhanced transcription when cloned upstream of the exogenous *Mycobacterium paratuberculosis* P$_{AN}$ promoter.

Recent results (31) demonstrated that *katG* expression is greatly activated in a *furA* knockout mutant, whereas overexpression is reduced by the FurA protein provided in *trans*. Thus, FurA may act as a *katG* repressor either directly (by interacting with a specific regulatory region upstream of *katG*) or indirectly (by repressing expression of a *katG* activator). Furthermore, the possibility that FurA controls catalase-peroxidase expression at the posttranscriptional level cannot be excluded. The identification of possible targets recognized by FurA is under investigation.

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