Responses of *Mycobacterium tuberculosis* Hemoglobin Promoters to In Vitro and In Vivo Growth Conditions[▽]

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The success of *Mycobacterium tuberculosis* as one of the dreaded human pathogens lies in its ability to utilize different defense mechanisms in response to the varied environmental challenges during the course of its intracellular infection, latency, and reactivation cycle. Truncated hemoglobins trHbN and trHbO are thought to play pivotal roles in the cellular metabolism of this organism during stress and hypoxia. To delineate the genetic regulation of the *M. tuberculosis* hemoglobins, transcriptional fusions of the promoters of the *glbN* and *glbO* genes with green fluorescent protein were constructed, and their responses were monitored in *Mycobacterium smegmatis* and *M. tuberculosis* H37Ra exposed to environmental stresses in vitro and in *M. tuberculosis* H37Ra after in vivo growth inside macrophages. The *glbN* promoter activity increased substantially during stationary phase and was nearly 3- to 3.5-fold higher than the activity of the *glbO* promoter, which remained more or less constant during different growth phases in *M. smegmatis*, as well as in *M. tuberculosis* H37Ra. In both mycobacterial hosts, the *glbN* promoter activity was induced 1.5- to 2-fold by the general nitrosative stress inducer, nitrite, as well as the NO releaser, sodium nitroprusside (SNP). The *glbO* promoter was more responsive to nitrite than to SNP, although the overall increase in its activity was much less than that of the *glbN* promoter. Additionally, the *glbN* promoter remained insensitive to the oxidative stress generated by H_2O_2, but the *glbO* promoter activity increased nearly 1.5-fold under similar conditions, suggesting that the trHb gene promoters are regulated differently under nitrosative and oxidative stress conditions. In contrast, transition metal-induced hypoxia enhanced the activity of both the *glbN* and *glbO* promoters at all growth phases; the *glbO* promoter was induced ~2.3-fold, which was found to be the highest value for this promoter under all the conditions evaluated. Addition of iron along with nickel reversed the induction in both cases. Interestingly, a concentration-dependent decrease in the activity of both trHb gene promoters was observed when the levels of iron in the growth media were depleted by addition of an iron chelator. These results suggested that an iron/heme-containing oxygen sensor is involved in the modulation of the trHb gene promoter activities directly or indirectly in conjunction with other cellular factors. The modes of promoter regulation under different physiological conditions were found to be similar for the trHbs in both *M. smegmatis* and *M. tuberculosis* H37Ra, indicating that the promoters might be regulated by components that are common to the two systems. Confocal microscopy of THP-1 macrophages infected with *M. tuberculosis* carrying the trHb gene promoter fusions showed that there was a significant level of promoter activity during intracellular growth in macrophages. Time course evaluation of the promoter activity after various times up to 48 h by fluorescence-activated cell sorting analysis of the intracellular *M. tuberculosis* cells indicated that the *glbN* promoter was active at all time points assessed, whereas the activity of the *glbO* promoter remained at a steady-state level up to 24 h postinfection and increased ~2-fold after 48 h of infection. Thus, the overall regulation pattern of the *M. tuberculosis* trHb gene promoters correlates not only with the stresses that the tubercle bacillus is likely to encounter once it is in the macrophage environment but also with our current knowledge of their functions. The in vivo studies that demonstrated for the first time expression of trHbs during macrophage infection of *M. tuberculosis* strongly indicate that the hemoglobins are required, and thus important, during the intracellular phase of the bacterial cycle. The present study of transcriptional regulation of *M. tuberculosis* hemoglobins in vitro under various stress conditions and in vivo after macrophage infection supports the hypothesis that biosynthesis of both trHbs (trHbN and trHbO) in the native host is regulated via the environmental signals that the tubercle bacillus receives during macrophage infection and growth in its human host.

Mycobacterium tuberculosis is a phenomenally successful pathogen, having evolved mechanisms that allow it to survive inside macrophages in granulomas that are characterized by severe hypoxia, a low pH, toxic nitrogen and oxygen species, and high CO_2 levels. *M. tuberculosis* not only appears to have the remarkable ability to adapt its metabolism to the hazardous environment of its intracellular niche but has also developed

efficient ways to persist during long periods of latency (12). Understanding the molecular mechanisms by which *M. tuberculosis* is able to adapt its metabolism to environmental changes and enhance its persistence within the host cells would, therefore, contribute significantly to the development of new strategies to combat tuberculosis. The genome of *M. tuberculosis* contains the *glbN* and *glbO* genes encoding the truncated hemoglobins (trHbs) trHbN and trHbO, respectively, which belong to a recently discovered family of hemoproteins that are widely distributed in eubacteria, cyanobacteria, protozoans, and plants (36, 39, 45, 55). The existence of two trHbs in *M. tuberculosis* that have low sequence similarity

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and distinct ligand binding properties and are expressed at different stages of growth in the native host (7, 38, 43) suggests that these oxygen-binding proteins may have different physiological functions. Recent studies in our laboratory (27, 42) and the laboratory of Ouellet et al. (40, 41) have indicated that trHbN of *M. tuberculosis* has a potent ability to detoxify nitric oxide (NO) and relieves the toxicity of NO and nitrosative stress (42) in *Escherichia coli* and *Mycobacterium smegmatis*. The high rate of NO oxidation catalyzed by oxygenated trHbN suggests that this may be one of the vital defense systems in *M. tuberculosis* (41, 42) for coping with the toxic effects of NO and nitrosative stress that may be important for allowing the intracellular survival of the bacterium in macrophages. This hypothesis is supported by the observation that expression of trHbN in an NO-sensitive mutant of *Salmonella* enhances the survival of the mutant both in vitro under nitrosative stress conditions and in vivo during macrophage infection (44).

The physiological role of trHbO in *M. tuberculosis* is not obvious at present; however, the presence of trHbO during all stages of growth in the native host (43) suggests that it may be important for cellular metabolism. This suggestion is supported by the presence of trHbO in *Mycobacterium leprae*, which has undergone drastic genome reduction and has retained only a minimum set of genes required for intracellular survival. Since they are aerobic, the mycobacterial cells would have to have a mechanism to collect the available O_2 and deliver it to the respiratory components of the cell to survive in granulomas. It has been suggested that trHbO is a membrane-associated protein, and it has been shown to interact with the CyoB subunit of the *E. coli* cytochrome *o* complex. This may be crucial for facilitating O_2 availability to the component(s) of the electron transfer chain (31, 43) in order to maintain the basal metabolism during hypoxia and latency. The oxygenated trHbOs of *M. tuberculosis* and *M. leprae* also display moderate NO-scavenging activity (2), suggesting that trHbO may be tailored so that it has functional diversity and is involved in both NO detoxification and aerobic respiration.

Unlike vertebrate hemoglobins, which have only oxygen transport and storage functions, the functions of trHbs are very diverse and thus cannot be generalized; e.g., the trHbN of *Chlamydomonas eugametos* is induced in response to active photosynthesis (8), the trHbN of *Nostoc commune* is expressed only under anaerobic conditions (20), etc. The distinct functions of the proteins having similar folds and the finding that structurally similar molecules can become further differentiated functionally by being expressed at different times in the development of an organism emphasize the role of regulation by the environment. The functions of trHbN and trHbO are now emerging as important features of the survival strategy of *M. tuberculosis*. Adaptation to the changing environments that *M. tuberculosis* encounters in the course of a successful infection must require complex regulation of gene expression, and therefore, a detailed study of the regulation of the genes encoding trHbN and trHbO in response to different environmental stimuli may provide insight into the relevance of these oxygen-binding proteins in the cellular metabolism, survival, and pathogenesis of *M. tuberculosis* in its macrophage host. Until now, there has been no study of the transcriptional regulation of either of the *M. tuberculosis* trHbs, and the tran-

scription machinery of this highly evolved pathogen still has not been thoroughly explored. In light of the function of trHbs, it seems logical to begin a study of the regulatory mechanisms governing the expression of trHb genes with an inquiry into the way that the genes respond to different environmental stresses and the macrophage environment. A study of the transcriptional responses of the genes to various stresses during growth would help us not only understand the regulation of gene expression but also establish the function of the encoded proteins.

In the present work, we conducted a systematic study of regulation of the trHb genes of *M. tuberculosis* by constructing transcriptional fusions with green fluorescent protein (GFP) and monitoring the promoter activity both in vitro under various stress conditions and in vivo after infection of THP-1 macrophages.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Unless otherwise noted, *M. smegmatis* mc²155 and *M. tuberculosis* H37Ra were grown in Middlebrook 7H9 medium or on Middlebrook 7H10 agar plates (Difco) supplemented with 0.05% Tween, 0.2% glycerol, and the OADC (10% bovine serum albumin fraction V, dextrose, catalase, oleic acid) enrichment for *M. tuberculosis*. All transformations in *E. coli* were performed with strain JM109. *E. coli* was grown in Luria broth (Difco) at 37°C in shaking cultures. For *E. coli* and mycobacteria, 100 and 50 μg/ml of hygromycin, respectively, were added to the medium when required.

Construction of transcriptional fusions. The transcriptional fusions were constructed by PCR amplification of a sequence encompassing positions −240 to 26 and positions −410 to 70 relative to the start codons of *glbN* and *glbO*, respectively, from *M. tuberculosis* H37Rv genomic DNA using primers that incorporated a 5′ XbaI site and a 3′ EcoRV site. The XbaI- and EcoRV-digested PCR products were ligated to XbaI- and EcoRV-cut pSC301 (9) from which the *SOD* promoter had been excised. pSC301b, the GFP read-through control vector, was constructed by digestion of pSC301 with XbaI and EcoRV to remove the *SOD* promoter, end filling with Vent polymerase, and religation. The constructs were electroporated into *M. smegmatis* mc²155 and *M. tuberculosis* H37Ra as previously described (54).

Determination of activities of transcriptional fusions. The *M. smegmatis* clones, transformed with the transcriptional fusions, were grown for 3 to 4 days and were then inoculated into fresh medium at an optical density at 600 nm (OD₆₀₀) of 0.05. The cultures were allowed to grow for another 8 to 10 h, resulting in an OD_{600} of around 0.5. The cells were then exposed to different physiological stresses, including 10 and 30 mM nitrite and 0.5 and 1.0 mM sodium nitroprusside (SNP) for nitrosative stress; 1 and 5 mM H_2O_2 for oxidative stress; 0.2, 0.3, and 0.4 mM 2,2′dipyridyl as an iron chelator; and medium whose pH was adjusted to 7 and 4. One hundred microliters of each culture was removed at an OD_{600} of 0.6 (early exponential phase), 1.2 (late exponential phase), and 2.0 (early stationary phase). The samples were diluted into 1 to 2 ml of phosphate-buffered saline (PBS), and the numbers of relative fluorescence units (RFU) were determined using a 490-nm excitation filter and a 520-nm emission filter with a spectrofluorometer (Perkin Elmer). The OD_{600} of each culture was determined with a UV-1601 Shimadzu spectrophotometer. The background fluorescence due to read-through transcription of the transcriptional fusion vector was determined by determining the number of RFU for strains that harbored the pSC301b plasmid and were subjected to the same conditions. The experiments with *M. tuberculosis* were done similarly. The RFU/OD_{600} values were plotted versus time. All measurements were obtained for triplicate cultures (means ± standard deviations) and were corrected for background fluorescence by subtracting the number of RFU for the control strain carrying pSC301b incubated under identical conditions.

Confocal microscopy. THP-1 cells were grown in RPMI 1640 containing 5% fetal bovine serum. In a 24-well tissue culture plate, 10^5 cells were seeded onto glass coverslips in RPMI 1640 containing 5% fetal bovine serum and then activated using 25 ng/ml phorbol myristate acetate (PMA) (Sigma) for 24 h. Infection was performed as previously described (14). After 4 h of infection, the cells were washed extensively with PBS to remove the extracellular bacteria. The

(A)

Mycobacterium tuberculosis
Mycobacterium bovis
Mycobacterium leprae
Mycobacterium avium
Mycobacterium sp. KMS
Mycobacterium sp.JLS
Mycobacterium sp. MCS
Mycobacterium ulcerans
Mycobacterium gilvum
Mycobacterium vanbaalenii
Mycobacterium smegmatis

glbN
Glutamine amidotransferase
Stress Response Protein
Pseudogene
Cynamide Hydratase
Short chain alcohol dehydrogenase
Methyl transferase
LprI
Fatty acylCoA reductase
Structural tRNA
LprL
Regulator (TetR family)

(B)

Mycobacterium tuberculosis
Mycobacterium bovis
Mycobacterium smegmatis
Mycobacterium avium
Mycobacterium sp. KMS
Mycobacterium sp.JLS
Mycobacterium sp. MCS
Mycobacterium ulcerans
Mycobacterium gilvum
Mycobacterium vanbaalenii
Mycobacterium leprae

glbO
α-amylase
HNH-like endonuclease
Response regulator receiver protein
α-amylase Psuedogene

(C)

Putative *glbN* promoter

5'-TT<u>GGCGGC</u>AATCAGCTTGTTACAGCT<u>GATAAT</u>CCGAGCGTGATCGCGACCTAACC**GGCAGG**-3'
　　-35　　　　　　　　　　　　　-10　　　　　　　　　　　　　　　　SD

Putative *glbO* promoter

5'-GG<u>CTGGCT</u>GGTCGGCCAC<u>GACAAA</u>TAGACCACAA**TGGAGG**GGATGGATA –3'
　　-35　　　　　　　-10　　　　　　　　　SD

FIG. 1. Schematic representation of the genomic organization of (A) the *glbN* gene and (B) the *glbO* gene. (C) In silico-predicted putative promoter sequences of the *glbN* and *glbO* genes. The −10 and −35 sequences are underlined, and the Shine-Dalgarno (SD) sequence is indicated by bold type.

cells were then fixed with 4% formaldehyde and visualized by confocal fluorescence microscopy.

Infection of THP-1 macrophages with *M. tuberculosis* and preparation of bacteria for FACS analysis. For fluorescence-activated cell sorting (FACS) analysis, THP-1 cell monolayers were established in 100-mm tissue culture petri dishes with 10^7 cells. After overnight incubation, the cells were activated using 25 ng/ml PMA for 24 h. The *M. tuberculosis* cells were prepared for infection by washing them three times with PBS under sterile conditions. The bacteria were then resuspended in 1 ml of PBS, and macrophages were infected using a multiplicity of infection of 1:20 in two duplicate sets. The cells were incubated at 37°C in 95% air-5% CO_2 for 4 h, after which they were extensively washed with PBS to remove the noningested bacteria. The infected macrophage monolayers were then incubated for 12, 24, and 48 h.

Each macrophage monolayer was washed five times with PBS. In vivo-grown mycobacteria were prepared using a previously described protocol (13). Briefly, the macrophage cells were harvested by using a scraper, collected by centrifugation (1,200 rpm for 10 min at 4°C), and then broken by 20 passages through a 23-gauge needle. Intact cells and nuclei were removed by pelleting them by centrifugation at 3,000 rpm for 5 min, and the supernatant containing bacteria was then centrifuged at 13,000 rpm for 5 min. The pellet obtained was resuspended in PBS for FACS analysis. The in vitro-grown mycobacteria were washed three times with PBS and resuspended in 1 ml of PBS by passage through a 23-gauge needle. For autofluorescence control, *M. tuberculosis* cells transformed with control vector pSC301b were prepared similarly.

Flow cytometry. Flow cytometric analysis was carried out using a Becton Dickinson FACScan and the Cell Quest software (Becton Dickinson). Data were collected for 10,000 individual particles per sample, with gating for size to eliminate interference due to potential clumping using the previously described procedure (13).

RESULTS

Genetic organization and promoter structure of *M. tuberculosis* trHbs. Conservation of gene order in prokaryotes has been considered one of the important predictors of gene function that helps in speculating about the probable function of a gene based on its positional counterparts or gene organization. The genomic organization around *glbN* (Fig. 1A) appeared to be similar only in the pathogenic members of the *M. tuberculosis* complex, and the nonpathogenic members had an altogether different genetic arrangement (Fig. 1A). Interestingly, the genomic organization of the *glbO* gene in various mycobacterial species was fairly well conserved (Fig. 1B), and 9 of the 11 species had identical loci flanking the *glbO* gene. Upstream intergenic regions of the *glbN* (228 bp) and *glbO* (143 bp) genes did not contain any obvious recognition sequences for mycobacterial stress response or oxygen-dependent transcriptional regulators. Analysis of the upstream region using the promoter prediction program available at http://www.fruitfly.org/seq_tools/promoter.html predicted the presence of putative promoter elements characterized by scores of 0.87 and 0.66, respectively (Fig. 1C). The transcription start sites were predicted to be 11 and 50 bp upstream of the translational start sites of *glbO* and *glbN*, respectively. The putative promoters

showed that a distinct Shine-Dalgarno sequence and probable -10 and -35 sites were present.

Transcriptional fusion of *M. tuberculosis* trHb gene promoters with the *gfp* gene. To study the transcriptional activity of the putative promoters of the *glbN* and *glbO* genes of *M. tuberculosis*, we constructed transcriptional fusions with the promoterless *gfp* gene in the *E. coli-Mycobacterium* shuttle vector pSC301 (9). The *glbN* and *glbO* transcriptional fusions were designated pSCNP and pSCOP, respectively. The *glbN*- and *glbO*-GFP promoter fusions exhibited low but distinct transcriptional activity in *E. coli*; however, their transcriptional responses were not studied in *E. coli* due to the great differences in the transcriptional machinery and regulation of the two organisms (1). Therefore, pSCNP and pSCOP were transformed into *M. smegmatis*, which has been widely used as a suitable heterologous host for studying transcriptional control of *M. tuberculosis* promoters (15, 23). The assay of the promoter activities in *M. smegmatis* using spectrofluorometry showed that there was significant up-regulation of the *glbN* promoter during the late exponential and stationary phases of growth, whereas the *glbO* promoter was constitutively active at a constant level throughout the growth cycle. The transcriptional activities of the trHb gene promoters were recorded following exposure to a variety of stress conditions and compared with the untreated control activities at early exponential phase (OD_{600}, 0.6), late exponential phase (OD_{600}, 1.2), and stationary phase (OD_{600}, 2.0) of growth.

Activities of *M. tuberculosis* trHb gene promoters in *M. smegmatis* under different environmental conditions. To study the implications of trHbs for intracellular growth and survival of *M. tuberculosis*, expression of the *glbN* and *glbO* promoters was first studied in vitro under conditions that simulated the major environmental challenges that the tubercle bacillus faces during its pathogenic stage, including various levels of oxidative and nitrosative stresses, pH challenge, and oxygen and nutrient limitation, in order to carry out aerobic metabolism.

The first stress faced by *M. tuberculosis* during infection is exposure to oxidizing agents, principally represented by the reactive oxygen intermediates (ROIs) and the reactive nitrogen intermediates (RNIs), produced by the activated macrophages. For induction of nitrosative stress, we used nitrite, which protonates to form HNO_2, which quickly dismutates to produce several species of nitrogen oxides, including NO (nitric oxide). There was a dose-dependent increase in *glbN* promoter activity in the presence of nitrite, with the levels of induction ranging from 1.3- to 1.5-fold with 10 mM nitrite and from 1.5- to 1.9-fold with 30 mM nitrite, at different growth phases (Fig. 2A). To evaluate whether the induction resulting from addition of nitrite was due to the combined effect of reactive nitrogen species generated during protonation of nitrite or due solely to release of NO during nitrite reduction, SNP, which specifically releases NO, was employed. The *glbN* promoter activity was induced 1.4- to 1.7-fold at all three growth phases after addition of both 0.5 and 1.0 mM SNP (Fig. 2B), suggesting that NO may be one of the key factors for modulating the *glbN* promoter activity.

The activity of the *glbO* promoter was enhanced in the presence of nitrite at all the growth phases in a more or less concentration-dependent manner (Fig. 2C). The level of induction (1.4-fold) was lower than that observed for the *glbN* promoter (1.5- to 1.9-fold). With SNP, a noticeable difference in the *glbO* promoter activity was observed only at the stationary phase, with only ~1.3-fold up-regulation (Fig. 2D). Addition of H_2O_2 for induction of oxidative stress did not result in any significant difference in the promoter activities of the two trHbs during the exponential phase of growth. However, the *glbO* promoter consistently showed moderate (~1.3-fold) up-regulation in the presence of H_2O_2 during the stationary phase (Fig. 3A and B).

Even if *M. tuberculosis* is able to block phagosome acidification, the block is not complete as there is slight decrease in the pH of the mycobacterial phagosomes (13). Therefore, the effect of culture pH on the transcriptional activity of the trHb gene promoters was investigated by using media in which the initial pH value was adjusted to 7 and 4. Both promoters were found to be independent of regulation by pH (data not shown).

It is generally accepted that the environment within the tuberculous granuloma is hypoxic (32), and extensive redirection of gene expression was noted in *M. tuberculosis* cultures in response to a brief period of oxygen limitation (11). Recent reports suggested that addition of transition metals could induce hypoxic genes under normoxia (5, 21, 26, 53). When we added 2 mM nickel chloride to the growth medium to induce hypoxia, there was ~1.7-fold induction of *glbN* promoter activity at the early and late exponential growth phases (Fig. 4A). In the case of the *glbO* promoter, the level of induction was higher and reached ~2.3-fold at the stationary phase (Fig. 4B). The induction of the trHb gene promoters was reversed when iron (20 μM $FeCl_3$) was added along with 2 mM nickel chloride to the growth medium, indicating that an iron/heme-containing factor(s) is involved in regulation of the transcriptional activity of the *glbN* and *glbO* promoters (Fig. 4A and B).

Inside the phagosomes and granulomas, the availability of nutrients and essential elements, especially iron (17) and magnesium (4), is greatly reduced. We examined the effects of diminished iron levels on the promoters of the trHbs by using the iron chelator 2,2′-dipyridyl at three different concentrations. A concentration-dependent decrease in the promoter activity was observed for both the trHb gene promoters. This effect of iron depletion was most pronounced during the stationary phase, when maximum down-regulation (twofold) was observed for the *glbN* promoter (Fig. 4C). A greater concentration-dependent decrease was observed for the activity of the *glbO* promoter compared to the activity of the *glbN* promoter, with values ranging from 1.7- to 2.3-fold (Fig. 4D).

Activities of trHb gene promoters in *M. tuberculosis* H37Ra. *M. smegmatis* has safely been used as a surrogate host for expression of genes from slow-growing pathogenic mycobacteria, but there are several promoters which do not follow the same pattern in *M. tuberculosis* and *M. smegmatis* (30). In addition to this, there are some differences in the patterns of the sigma factors of the two organisms (33). Since the most homologous system available for *M. tuberculosis* H37Rv is *M. tuberculosis* H37Ra, we also evaluated trHb promoter activity in this host. The promoter-GFP fusions were transformed in *M. tuberculosis* H37Ra, where the activities exhibited the same pattern that they exhibited in *M. smegmatis*, with the *glbN* gene promoter up-regulated at stationary phase and the *glbO* gene promoter constitutively active (data not shown). The trHb gene promoter activities were assayed under all stress condi-
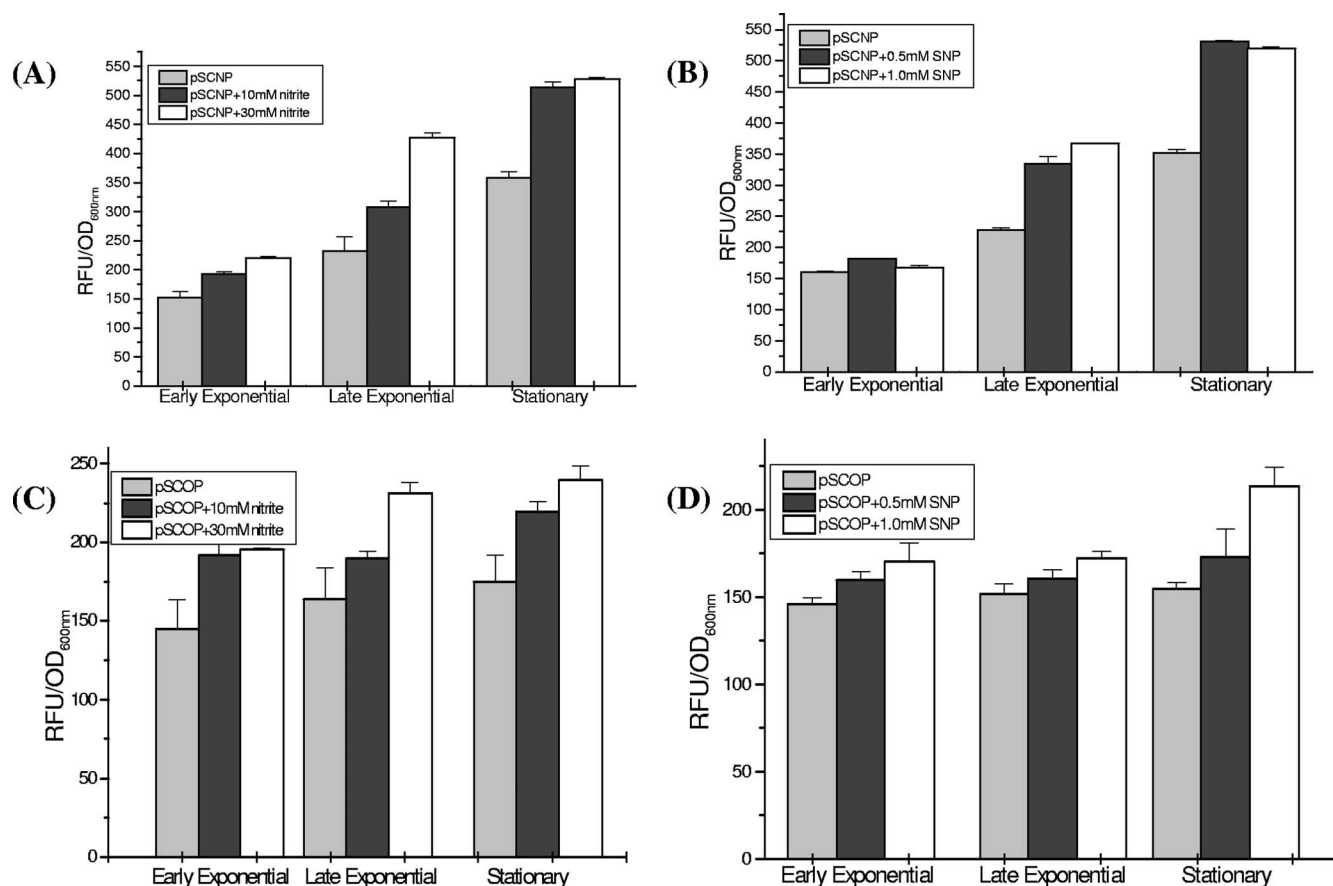
FIG. 2. Transcriptional activities of the trHb gene promoters in *M. smegmatis* under nitrosative stress conditions. *M. smegmatis* mc^{2}155 cells transformed with pSCNP or pSCOP were grown to an OD_{600} of around 0.5, and then 10 or 30 mM sodium nitrite or 0.5 or 1.0 mM SNP was added. GFP fluorescence was measured at OD_{600} of 0.6 (early exponential phase), 1.2 (late exponential phase), and 2.0 (early stationary phase). (A and B) *glbN* promoter activities at different growth phases of *M. smegmatis* mc^{2}155 (A) in the presence of nitrite and (B) in the presence of SNP. (C and D) *glbO* promoter activities at different growth phases of *M. smegmatis* mc^{2}155 (C) in the presence of nitrite and (D) in the presence of SNP. Statistical significance was established using a *P* value of <0.05.

tions employed for *M. smegmatis*. The results obtained showed that the patterns were very similar and that there were similar changes in the trHb gene promoter activities. Representative results for trHb gene promoter activities in the presence of nitrite and H$_2$O$_2$ are presented here. The results showed that the *glbN* promoter behaves more or less similarly in the two mycobacterial species (Fig. 5A and C). The *glbO* promoter, however, showed slightly higher levels of induction under both nitrosative and oxidative stress conditions in *M. tuberculosis* than in *M. smegmatis* (Fig. 5B, 5D).

Activities of *M. tuberculosis* trHb promoters during intracellular infection. In vitro studies of transcriptional regulation of the trHb gene promoters clearly indicated that the responses of the promoters of the *glbN* and *glbO* genes were distinct under different stress conditions, suggesting that the promoters are relevant under in vivo growth conditions. Additionally, for the function of trHbN and trHbO to be relevant to the intracellular growth of *M. tuberculosis*, it is important to determine if these compounds are synthesized during the intracellular phase. The observation that GFP is efficiently expressed in mycobacteria and the observation that fluorescent bacteria can be directly observed in infected macrophages (13) suggested

that such a strategy may be applicable to studies of gene induction by mycobacteria in host cells. To study the intracellular expression of the hemoglobin genes, PMA-activated THP-1 cells were used, which have been reported to closely model the primary human alveolar macrophages (46) that are the critical growth niche for intracellular *M. tuberculosis* in the lungs (29). The PMA-activated THP-1 cells were infected with *M. tuberculosis* H37Ra carrying the transcriptional fusion of the *glbN* and *glbO* genes. Confocal microscopy of the infected THP-1 cells showed that there was distinct fluorescence in the phagocytosed *M. tuberculosis* H37Ra cells carrying *glbN* and *glbO* transcriptional fusions (data not shown).

M. tuberculosis cells harboring *glbN* or *glbO* promoter fusions were used to infect PMA-activated THP-1 cells, and at 4, 12, 24, and 48 h postinfection, THP-1 cells were lysed and the intracellular bacteria were harvested for analysis by flow cytometry. Two peaks at which the low-fluorescence fraction was composed mainly of cell debris were observed, as has also been observed by Dhandayuthapani et al. (13) for *Mycobacterium bovis*. The peak showing a higher level of fluorescence consisted of intact bacterial cells isolated from macrophages. The overall maximum for this peak was much lower than that ob-
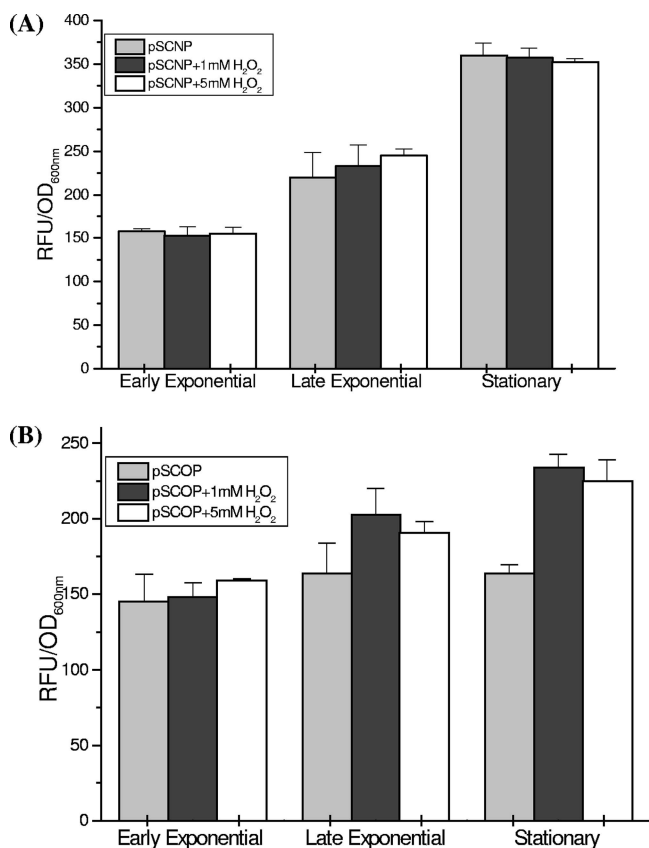
FIG. 3. Transcriptional activities of the trHb gene promoters in *M. smegmatis* under oxidative stress conditions. (A) *glbN* and (B) *glbO* promoter activities at different growth phases of *M. smegmatis* mc^{2}155 in the presence of 1 and 5 mM H_2O_2. Statistical significance was established using a *P* value of <0.05.

tained with the bacteria used to infect the macrophages, which probably reflected differences in the total numbers of particles in the samples.

The FACS analysis of bacteria collected immediately after infection (for 4 h) revealed the presence of highly fluorescent cells, suggesting that both the trHb gene promoters were active. The distribution of fluorescence for the bacteria grown in macrophages was found to be similar to the distribution of fluorescence for the in vitro-grown bacteria. Time course FACS analysis of in vivo-grown *M. tuberculosis* after 12, 24, and 48 h of intracellular growth indicated that functionally active trHb gene promoters were present during these periods. The mean fluorescence intensities of mycobacteria collected at various time points during infection relative to the mean fluorescence intensities of mycobacteria grown in vitro showed that there was a slight but consistent increase in the activity of the *glbN* promoter from 4 to 48 h (Table 1). The *glbO* promoter showed a much more drastic change; the mean fluorescence intensities of the in vivo- and in vitro-grown mycobacteria were more or less similar until 24 h after infection, but the level of fluorescence of bacteria isolated from infected macrophages was found to be almost twice that of the in vitro-grown bacteria after 48 h of infection (Table 2). Overall, the in vivo studies of transcriptional regulation of the *M. tuberculosis* trHb gene pro-

moters clearly indicated the presence of active *glbN* and *glbO* promoters whose activity increased after continued growth in the intramacrophage environment. The results, therefore, indicate that there is a steady expression of both trHbs and that both trHbs may be present during macrophage infection.

DISCUSSION

In *M. tuberculosis*, during its intracellular phase, there is coordinated gene expression in response to signals associated with various stages of the infection cycle. Although a number of virulence genes and their response regulators have recently been identified (19, 56), the mechanisms governing the intracellular survival of this organism and its persistence within the human host are poorly understood. Global analysis of gene expression in several pathogenic bacteria, including *M. tuberculosis*, has revealed that large-scale changes occur upon in vitro exposure to the environmental conditions that simulate the intracellular milieu (28, 35, 49). trHbN and trHbO are produced by *M. tuberculosis* at different stages of growth (7, 38, 43), and it has been proposed that they confer an in vivo survival advantage by evading toxic effects of reactive oxygen and nitrogen species in the hypoxic environment of macrophages due to their distinct ability to scavenge reactive oxygen and nitrogen species (44) and trap oxygen with high efficiency, which may contribute significantly to overcoming the intracellular host defense mechanisms.

Mycobacterial genome sequence analysis has indicated that trHbs may be widely distributed among pathogenic and nonpathogenic species. Interestingly, the trHbO-encoding gene (*glbO*) has been identified in all mycobacterial species, including *M. leprae*, and its genetic organization is quite conserved across various species compared to *glbN*, suggesting that trHbO might be involved in a more basic and/or crucial function needed for mycobacterial cellular metabolism. In contrast, the genomic organization of *glbN* is not very well conserved across mycobacterial species. It is quite possible that trHbN may have distinct roles in different mycobacterial species, as has recently been shown for the function of trHbN of *M. smegmatis* and *M. tuberculosis* (27); thus, the conservation of the *glbN* gene organization among different species of mycobacteria may not be very relevant.

The tubercle bacillus encounters the potentially hypoxic and hostile environment of the granuloma, which is enriched with various reactive oxygen and nitrogen species, by using specific and general stress responses involving regulation of the components of its defense mechanisms. The transcriptional activities of the *glbN* and *glbO* promoters during aerobic growth of *M. smegmatis* and *M. tuberculosis* were consistent with the protein expression pattern observed for trHbN and trHbO in *M. bovis* (38, 43). The *glbN* promoter was up-regulated both by the general nitrosative stress inducer nitrite and by a specific NO releaser (SNP), suggesting that there is induction of trHbN biosynthesis during exposure to NO and other reactive nitrogen species. We have previously demonstrated that trHbN of *M. tuberculosis* has a potent NO dioxygenase activity that converts toxic NO into harmless nitrate in the presence of oxygen and relieves toxicity due to NO and nitrosative stress (42). It has been proposed that because of its high oxygen affinity, trHbN
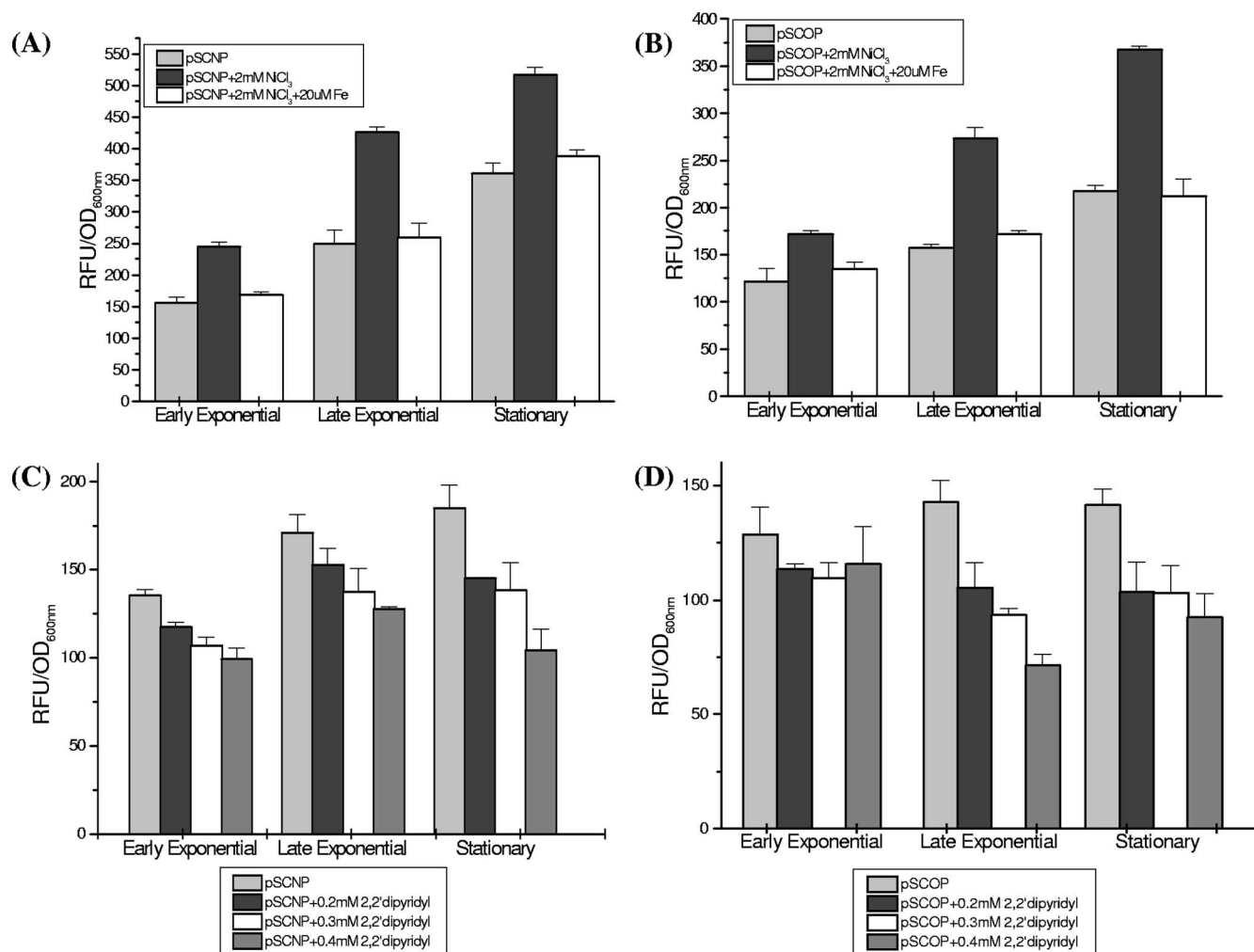
FIG. 4. Transcriptional activities of the trHb gene promoters under transition metal-induced hypoxia and iron depletion conditions. (A) *glbN* and (B) *glbO* promoter activities at different growth phases of *M. smegmatis* mc^{2}155 in the presence of 2 mM NiCl$_3$ and/or 20 μM FeCl$_3$ and (C) *glbN* and (D) *glbO* promoter activities at different growth phases of *M. smegmatis* mc^{2}155 in the presence of different concentrations of the iron chelator 2,2′dipyridyl. Statistical significance was established using a P value of <0.05.

retains bound oxygen and ensures availability of a critical level of oxygen to carry out NO detoxification in the hypoxic and NO-enriched environment of macrophages, ensuring intracellular survival of *M. tuberculosis*. The observed regulation behavior can be correlated to the previously observed function of the encoded trHbN, which detoxifies NO and, therefore, should be available to the mycobacteria whenever toxic levels of NO are present. No change in *glbN* promoter activity was observed in *M. smegmatis* or *M. tuberculosis* under oxidative stress conditions or after a change in pH, which ruled out the possibility that there was regulation of the trHbN gene by these conditions.

The response of the *glbO* promoter in the mycobacterial hosts *M. smegmatis* and *M. tuberculosis* appeared to be very different from that of the *glbN* promoter under nitrosative and oxidative stress conditions. The transcriptional activity of the *glbO* promoter increased under nitrosative stress conditions, but only to a level lower than that of the *glbN* promoter. During stationary phase, the *M. tuberculosis glbO*

promoter exhibited nearly 1.3-fold induction in the presence of SNP, which is very similar to the level observed for the *glbO* promoter of *M. leprae* (15). The promoter activity of *glbO* was also enhanced under oxidative stress conditions, which suggests that the activity of the *glbO* promoter might be modulated by the cumulative effect of general stress. trHbO seems to be required for aerobic respiration (31, 43), and therefore, its levels are increased by the general stresses that the bacteria come across once they are inside phagosomes, which include RNIs, ROIs, and hypoxia. It has been proposed that in *M. leprae* trHbO has a dual function, respiration and NO detoxification (15, 50, 51). Such a role for *M. tuberculosis* trHbO has not been observed, but the fact that *M. tuberculosis* trHbO displays a low level of NO dioxygenase activity in *E. coli* and the fact that its promoter is induced under both oxidative and nitrosative stress conditions during stationary phase suggest that it might also have functional diversity very similar to that of trHbO of *M. leprae*. Additionally, a trHbN knockout strain of *M. bovis*
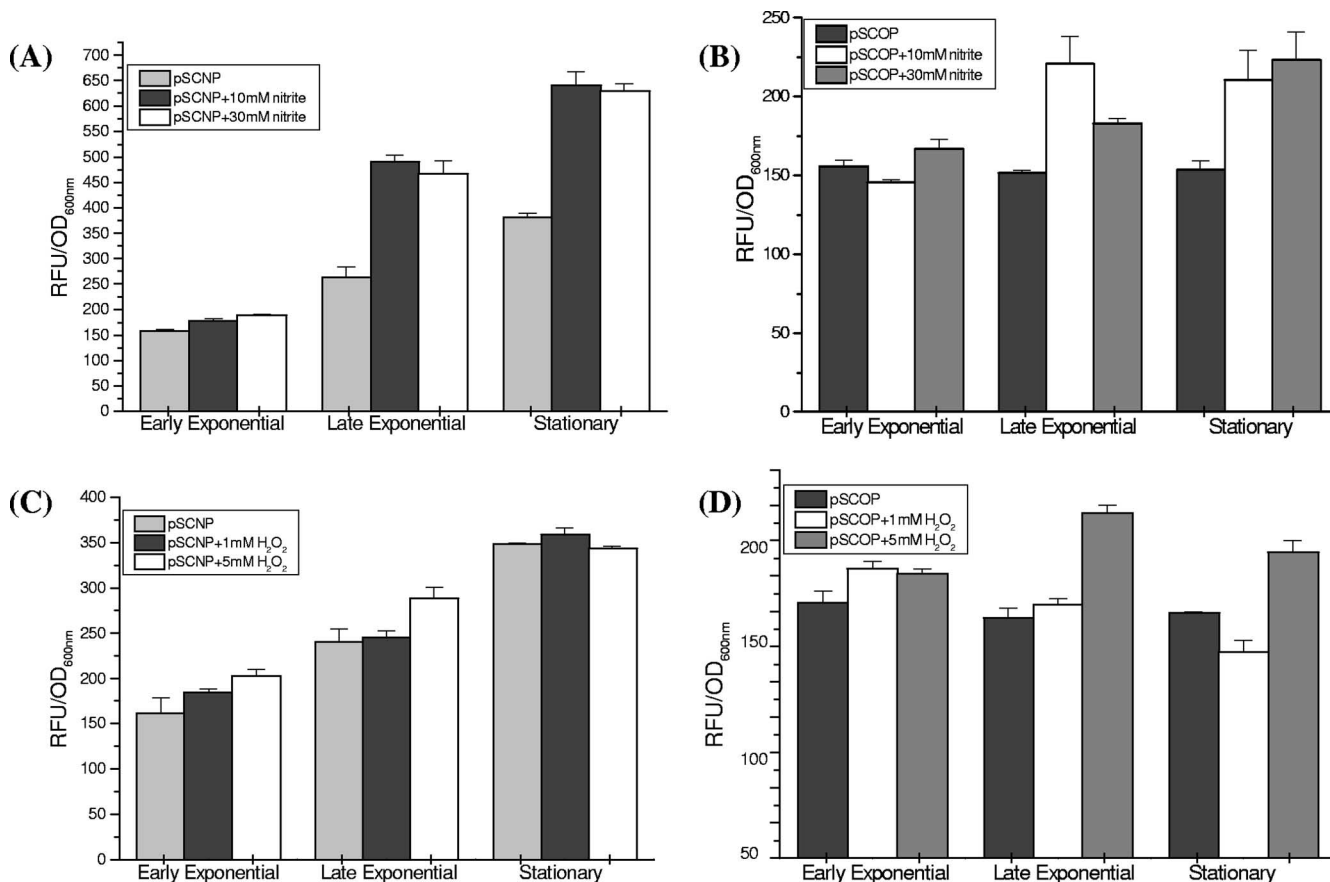
FIG. 5. Transcriptional activity of the trHb gene promoters in *M. tuberculosis* H37Ra under nitrosative and oxidative stress: (A) *glbN* and (B) *glbO* promoter activities at different growth phases of *M. tuberculosis* H37Ra in the presence of 10 and 30 mM sodium nitrite and (C) *glbN* and (D) *glbO* promoter activities at different growth phases of *M. tuberculosis* H37Ra in the presence of 1 and 5 mM H_2O_2. Statistical significance was established using a *P* value of <0.05.

retained NO uptake activity, albeit with lower efficiency, which might be attributed to the presence of trHbO (41).

In contrast to the results under oxidative and nitrosative stress conditions, the transcriptional activities of both the *glbN* and *glbO* promoters increased under transition metal-induced hypoxia. It has been observed that transition metals, like nickel or cobalt, induce a number of hypoxic genes under normoxic conditions (5, 21, 53) by serving as substrates for ferrochelatase and being incorporated into protoporphyrin IX in place of iron (48). Nickel-substituted hemoproteins, therefore, do not bind

oxygen and cannot function as oxygen sensors. Addition of nickel chloride induced the *glbO* promoter to the highest level observed for this promoter under all the conditions evaluated. These observations suggest that there is a hemosensor in mycobacteria that may up-regulate the activity of the trHb gene promoters. This suggestion was substantiated by a decrease in the up-regulation of the *glbN* and *glbO* promoters when iron, which is known to reverse the effect of addition of nickel by competing with ferrochelatase, was included along with nickel (26).

TABLE 1. Flow cytometric analysis of *M. tuberculosis* carrying the *glbN* transcriptional fusion

Time postinfection (h)	Fluorescence intensity (mean ± SE) of *M. tuberculosis* with pSCNP[a]	
	Grown in vitro	Grown in vivo
4	49.73 ± 0.35	45.57 ± 0.32
12	48.30 ± 0.31	48.46 ± 0.25
24	48.95 ± 0.28	49.82 ± 0.23
48	50.00 ± 0.41	61.44 ± 0.52

[a] The *M. tuberculosis* cells carried the *glbN* transcriptional fusion (pSCNP) and were harvested from shake flasks (grown in vitro) or infected THP-1 macrophages (grown in vivo). Three independent experiments were carried out, each in duplicate. The data are from one representative experiment.

TABLE 2. Flow cytometric analysis of *M. tuberculosis* carrying the *glbO* transcriptional fusion

Time postinfection (h)	Fluorescence intensity (mean ± SE) of *M. tuberculosis* with pSCOP[a]	
	Grown in vitro	Grown in vivo
4	23.41 ± 0.20	25.00 ± 0.32
12	29.82 ± 0.13	29.79 ± 0.16
24	28.90 ± 0.22	29.84 ± 0.03
48	23.41 ± 0.27	42.41 ± 0.44

[a] The *M. tuberculosis* cells carried the *glbO* transcriptional fusion (pSCOP) and were harvested from shake flasks (grown in vitro) or infected THP-1 macrophages (grown in vivo). Three independent experiments were carried out, each in duplicate. The data are from one representative experiment.

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In vertebrates, hemoproteins have been reported to act as oxygen sensors (5, 18, 22, 34, 52), and recently, heme has also been shown to be involved in regulating transcription of oxygen-responsive genes in *Saccharomyces cerevisiae* (25, 26). In fact, the heme-containing proteins DosS and DosT of *M. tuberculosis* have been shown to function as redox and hypoxia sensors, respectively (24). The concentration-dependent decrease in the activity of both trHb gene promoters after depletion of iron, further indicating involvement of an iron homeostasis regulator that may modulate the activity of the trHb gene promoters directly or indirectly by regulating general response regulators of hypoxia in mycobacteria. The overall responses of the trHb gene promoters in *M. tuberculosis* H37Ra and *M. smegmatis* were more or less similar under all the stress conditions used, which suggests that at least these two promoters behave in a similar manner in the two mycobacterial species and, therefore, might be regulated by components which are common to both systems.

The levels of induction of the trHb promoters ranged from 1.2- to 2.3-fold under the different growth conditions, which is quite a significant change at the protein level considering that GFP does not require any substrate and, therefore, does not require an amplification step for visualization of fluorescence. Most studies examining gene regulation in mycobacteria have shown a similar level of induction; for example, 11 of 12 *Mycobacterium marinum* promoters known to be induced exhibited 1.4- to 3.4-fold GFP induction (3). The trHb gene promoters were found to be maximally responsive to RNIs, iron depletion, and hypoxia. The multicellular structure within which the tubercle bacillus resides is also known to express NOS2 and to produce RNIs in situ in tuberculosis tissues of infected mice and humans (6, 16, 47). Apart from oxidative and nitrosative stresses inside the phagosomes and granulomas, the availability of nutrients and essential elements may be reduced (4, 17). Therefore, all these stresses are likely to be biologically relevant signals encountered by *M. tuberculosis* in vivo, which the tubercle bacillus then exploits as signals for regulation of the expression of specific genes that promote survival in the host.

The pattern of gene expression is modified for adaptation to an intracellular environment, as has been shown previously for *Salmonella* and *Listeria*, both of which are intracellular pathogens (37). By analogy, similar mechanisms are expected to be operative in *M. tuberculosis*. Therefore, identification of the genes which are expressed or up-regulated within the host cells would contribute to our understanding of the pathogenicity of this organism. We showed here for the first time using confocal microscopy that the trHb gene promoters were active during intracellular growth. FACS analysis showed that the *glbN* promoter exhibited much higher activity than the *glbO* promoter, and the *glbN* promoter was found to be active after intracellular growth for about 48 h, with a slow but steady increase in activity. However, we cannot rule out the possibility of further up-regulation of the *glbN* promoter after periods of infection longer than those studied here. The *glbO* promoter activity, like the in vitro expression pattern, remained at a steady-state level up to 24 h postinfection and, interestingly, increased ~2-fold after 48 h of infection. During the course of infection, there are several changes in the macrophage environment due to the release of cytokines in a time-dependent manner (for

example, NF-κB [14]), and there is activation of various protection mechanisms (for example, generation of ROIs and RNIs against the *Mycobacterium*). Therefore, the increase in *glbO* promoter activity might be due to induction of some host-specific factor in *Mycobacterium* itself or might be due to variation in the host environment. Most physiological studies have stressed the importance of trHbN for the survival of *M. tuberculosis* inside the host; however, the specific up-regulation of the trHbO promoter during growth inside macrophages places it on center stage along with trHbN. As previously observed (10), the activity of the *SOD* promoter, used as control, decreased significantly after just 4 h of intracellular growth of *M. tuberculosis* cells carrying pSC301 (data not shown). This suggests that the stresses that the *Mycobacterium* cells face during growth inside macrophages keep the trHb gene promoters active and that there might be a requirement for constant expression of both of the trHb proteins during intracellular growth of the *Mycobacterium*.

Together, the in vitro and in vivo studies in this work provide a comprehensive picture of the regulation of *M. tuberculosis* hemoglobin genes. The regulation of each promoter correlates not only with the stresses that the tubercle bacillus is likely to encounter once it is in the macrophage environment but also with our current knowledge of the functions of the promoters. *M. tuberculosis* is a phenomenally successful pathogen, having evolved mechanisms to precisely regulate the expression of proteins that allow it to survive in the ever-changing macrophage environment. To date, all the speculation about the functions and importance of the trHbs has been centered on the information obtained from studies conducted in vitro. The present study demonstrated for the first time expression of trHbs in *Mycobacterium* during growth inside macrophages. The specific up-regulation of the *glbO* promoter during growth inside macrophages reinforces the importance of this promoter for the survival of the mycobacterium. The in vivo studies presented here strongly indicate that the trHbs of *M. tuberculosis* are required and, thus, important in the natural habitat of the bacterium.

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