**Mycobacterium tuberculosis** is a slow-growing pathogen and is characterized by a low content of RNA per unit of DNA. rRNAs represent a major proportion of the total RNA pool, and the entire requirement for rRNA is met by transcription from a single **rrn** operon that is driven by two promoters, P1 and P3. This study attempted to analyze the specific role of the **rrn** promoter in determining the characteristically low levels of RNA in **M. tuberculosis**. For this purpose, the activity of the **M. tuberculosis** **rrn** promoter as a function of the growth rate was studied by **rrn-lacZ** promoter fusion, hybridization, and primer extension analysis in **M. smegmatis**. **rrn** promoter signals were faithfully recognized in **M. smegmatis** cultures harboring the **rrn-lacZ** promoter construct. In **M. smegmatis** cultures that displayed doubling times varying between 3.06 and 6.5 h, β-galactosidase activity increased sixfold in proportion to the growth rate (μ). There was a corresponding increase in the amount of **lacZ**-specific mRNA, while the plasmid copy number remained essentially unchanged. For any given μ, the P3 promoter was twofold more efficiently utilized than the P1 promoter. Since both promoters of the **M. tuberculosis** **rrn** operon are regulatable as a function of growth rate in **M. smegmatis** cultures, it is implied that the inherent structure or sequence of the **rrn** promoter per se is not primarily responsible for the observed lack of modulation of RNA synthesis in **M. tuberculosis**.

**Mycobacterium tuberculosis** is the causative agent of tuberculosis and is characterized by slow growth. The study of ribosome regulation is extremely relevant to understanding the molecular basis of the slow growth of this organism, since protein synthesis, so critical to growth, is dependent on ribosomes. **M. tuberculosis** cultures contain small amounts of RNA per unit content of DNA; the total RNA content varies only twofold between stationary-phase cultures and actively growing cells (38). The reasons underlying this lack of responsiveness can be addressed by directly analyzing rRNA transcriptional activity since rRNA comprises the majority (~80%) of the total RNA pool of a mycobacterial cell. The production of rRNA is determined by the number of **rrn** operons, the number of promoters, the nature of the promoter elements, and the efficiency with which the operons are transcribed. Since rRNAs represent a relatively stable population, breakdown is less likely to constitute a major regulatory mechanism and the regulation of RNA synthesis is expected to occur at the level of RNA chain initiation. In earlier studies from our laboratory, we demonstrated that fast-growing (**M. smegmatis**) and slow-growing (**M. tuberculosis**) mycobacteria follow a similar pattern of bacterial growth comprising the log, logarithmic, and stationary phases, with maximum rRNA levels found during the logarithmic phase of growth (7). The **M. tuberculosis** **rrn** operon is driven by twin promoters designated P1 and P3 (21); transcription start points (tsp), an RNase III-processing site, and the +1 of mature 16S rRNA were mapped in our laboratory (38). In **Escherichia coli**, rRNA synthesis is a rate-limiting step in ribosome production since r-protein expression is regulated by feedback mechanisms sensitive to the rRNA concentration (31). In several bacteria including **E. coli**, the number of ribosomes varies linearly with the growth rate, μ, over a range of conditions. This phenomenon has been termed growth rate-dependent control (GRDC) of ribosome synthesis, and it serves to maintain the cellular pool of ribosomes at a level commensurate with the requirement of the cell for protein synthesis at all times (18). GRDC of rRNA biosynthesis has been most extensively studied in **E. coli** and has been shown to occur at the level of **rrn** expression (2, 12, 16, 17, 24, 30, 34). The exact mechanism by which GRDC is attained is still under active investigation, although a large body of evidence implies a role for ppGpp and/or some translation-linked event (12). Since the **rrn** operon of **M. tuberculosis** is expressed from dual promoters, as in other eubacteria including **E. coli** (12) and **Bacillus subtilis** (35), we asked whether regulatory mechanisms operating in **E. coli** and **B. subtilis**, such as GRDC, may be applicable to **M. tuberculosis** **rrn** promoters. This study was designed to analyze the ability of the **M. tuberculosis** **rrn** promoters to respond to variations in the growth rate. To determine if the rRNA promoter sequence and structure per se impose any constraints on their usage, their activity was analyzed in **M. smegmatis**, a mycobacterial species often used as a surrogate host to study **M. tuberculosis** gene expression. We report (i) fidelity in usage and in the differential activity of the **M. tuberculosis** **rrn** P1 and P3 promoters and (ii) GRDC of the **M. tuberculosis** **rrn** promoters in cultures of **M. smegmatis** bearing **rrn-lacZ** constructs. The minimal role of the **M. tuberculosis** **rrn** promoter per se in determining the slow growth of **M. tuberculosis** is discussed.

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**Materials and Methods**

**Strains and plasmids.** Jack Crawford, Centers for Disease Control and Prevention, Atlanta, Ga., provided **M. smegmatis** LR222. A. K. Tyagi, University of Delhi South Campus, New Delhi, India, generously provided promoter selection vectors pSD7 and pSD5B.

**Construction of rrn-cat and rrn-lacZ fusions.** For the construction of the **rrn-lacZ** promoter fusion (Fig. 1), the **rrn** upstream sequence (816 bp) was amplified by inverse PCR (37) and a ~650-bp fragment was cloned into a XhoI site located upstream of the **lacZ** gene in pSD5B (26). The ligation mixture was...
FIG. 1. (A) Schematic representation of the *M. tuberculosis* *rrn* promoter. The top line shows the genomic organization of the *rrn* operon in *M. tuberculosis* (not to scale). The middle line shows the *PstI* fragment spanning the *rrn* promoter region. The region encompassed by the *PstI* and *Sau3AI* sites (mapping downstream of +1 of 16S rRNA) was amplified by inverse PCR as detailed previously (37) and cloned in pGEM3Zf1 to generate pAV16S.2. tsp a and c represent the start points and direction of transcription from the P1 and P3 *rrn* promoters, respectively, and are denoted by thin and thick arrows, respectively; the nomenclature of P1 promoter for tsp a and P3 promoter for tsp c corresponds to that by Gonzalez-y-Merchand et al. (21). d and +1 represent the experimentally determined RNase III-processing site and the start of the mature transcript, respectively (37). The lower part of the figure represents the restriction fragments cloned in promoter fusion vectors pSD5B (26) in the right (pSD5B.16SR) and wrong (pSD5B.16SW) orientations. (B) Nucleotide sequence of the *rrn* promoter of *M. tuberculosis*. The numbers on the right are according to Cole et al. (11) for the *M. tuberculosis* genome. The sequence of the PCR-amplified fragment including the promoter region, +1 mature 16S rRNA start site, and 78 bp of coding region is shown. tsp a and c and their respective promoters are indicated by arrowheads and highlighted boxes, respectively. The experimentally determined RNase III recognition sequence is highlighted and the cleavage site ‘d’ is indicated (37). The location and orientation of the primer-annealing sites are indicated by arrows. The *Sau3AI* sites mark the fragment that was cloned in plasmid pSD7. (C) Comparison of the primer-annealing regions in *M. tuberculosis* (*Mt*) and *M. smegmatis* *rrnA* (*Msm A) and *rrnB* (*Msm B) operons. The sequence of the RNA-like strand is shown. Identical residues are marked by vertical lines.
electroporated into E. coli, and the transformants were plated on Luria-Bertani agar containing kanamycin (25 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Cloning of the rrn promoter in the right orientation (pSD5B.16SR) without respect to lacZ produced colonies, and cloning in the left orientation (pSD5B.16SW) produced white colonies. Plasmid DNA was isolated from the transformants and electroporated into M. smegmatis to generate strains containing promoter-fusion constructs with the rrn promoter cloned in both orientations.

**Culture conditions.** M. smegmatis was maintained on Lowenstein-Jensen medium. A loopful was inoculated into 10 ml of Youmans and Karlsons (YK) liquid medium containing kanamycin (25 μg/ml) and Tween 80 (0.2%) and supplemented with 0.5% glycerol (YKKTG) in a 50-ml conical flask at 37°C with shaking. After growth to an optical density at 600 nm (OD600) of 0.04 to 0.06 and incubation with shaking at 37°C, to establish the starting inoculum, either pSD5B.16SR or pSD5B.16SW to an initial optical density at 600 nm (OD600) of 0.04 to 0.06 and incubated with shaking at 37°C. To establish the growth curve and growth rates, aliquots were taken every 4 h and the OD600 was measured.

**Results.**

In a preliminary experiment, a ~310-bp Sau3AI fragment (Fig. 1A) mapping between -233 and +74 relative to the +1 of mature 16S rRNA and containing P1 and P3 rrn promoters was cloned into the promoter selection vector pSD7 (13) to generate pSD7.16S. This fragment supported extremely high chloramphenicol acetyltransferase activity (16,665 pmol/min/mg of protein) in M. smegmatis (data not shown) in comparison to the relatively low activity observed for randomly cloned promoters of M. tuberculosis (5 to 2,500 pmol/min/mg of protein) in M. smegmatis, with the majority of promoters displaying activity in the range of 5 to 100 pmol/min/mg of protein (13).

**Growth rate dependence of the rrn promoter(s) of M. tuberculosis.** For the analysis of the rrn promoter, a ~650-bp rrn promoter-containing fragment was subcloned upstream of the lacZ reporter gene in shuttle plasmid vector pSD5B, a low-copy-number plasmid that is maintained at ~3 copies per mycobacterial cell (32) (Fig. 1A). Blue and white colonies of M. smegmatis transformants were obtained on a Luria-Bertani agar plate containing X-Gal and 20 μg of kanamycin per ml. When cloned in the right orientation (pSD5B.16SR), the rrn promoter drove β-galactosidase expression, yielding blue colonies, whereas when cloned in the wrong orientation (pSD5B.16SW), white colonies were obtained, clearly indicating that the promoter was functional in one direction only. M. smegmatis cells carrying rrn-lacZ fusions were grown as shake cultures in YKK medium (supplemented with 0.01 to 1% glycerol) at various growth rates and were assayed for β-galactosidase activity. The growth curves followed the expected pattern and yielded a range of growth rates (μ = 0.153 to 0.327), which were required to study the growth rate-dependence of rrn promoter (Fig. 2). The generation time of M. smegmatis cultures corresponding to the growth rates of 0.153 to 0.327 was in the range of 6.49 h to 3.06 h, respectively. The rrn promoter, when cloned in the right orientation (pSD5B.16SR), gave the steep positive slope of activity with increasing growth rate which is characteristic of GRDC (Fig. 3A). M. smegmatis carrying the rrn promoter cloned in the wrong orientation (pSD5B.16SW) showed negligible activity over a range of growth rates (data not shown). The presence of the fusions used in this study did not appear to impose any metabolic load, since there were no differences in the growth rates of cultures containing or not containing the plasmids.

**Variation of β-galactosidase transcript levels with growth rate.** To confirm that the growth rate-dependent response seen...
for M. tuberculosis rrn promoters cloned into pSD5B reflected the transcriptional activity of the promoters, the level of lacZ mRNA at different growth rates was determined by dot blot hybridization of RNA isolated from M. smegmatis cultures harvested at the same time point at which β-galactosidase activity was measured. The level of lacZ mRNA in cells carrying the rrn promoter cloned in the right orientation (pSD5B.16SR) increased in a growth rate-dependent manner. In contrast, hybridization with the kanamycin probe did not increase with an increase in μ for either pSD5B.16SR or pSD5B.16SW (Fig. 3B). These findings confirmed that (i) the increase in β-galactosidase activity with an increase in growth rate was due to enhanced transcriptional activity of the M. tuberculosis rrn promoter and (ii) the presence of the rrn promoter did not modulate kan gene transcription from the same plasmid.

Association of growth rate with plasmid copy number. Because a plasmid system was used to study rrn-lacZ transcription, we considered the possibility that fluctuations in plasmid copy number with different growth rates would present as a growth rate-dependent response. Others have found that growth conditions and strengths of inserted promoters can significantly affect the copy number (1, 36). Therefore, an estimate of the amount of plasmid DNA per unit of total cellular protein was obtained for promoter-fusion clones containing the rrn promoter as described in Materials and Methods. It is clear that the amount of plasmid DNA remained essentially unaltered as the growth rate increased; thus, it could not account for the increase seen in lacZ mRNA and β-galactosidase specific activity (Fig. 3C).

Promoter fusion studies have proven particularly useful in demonstrating that with an increase in growth rate, E. coli rrn promoter-directed transcription and translation of the reporter gene also increases (20, 27). Although reporter technology for the analysis of promoter activity is very extensively used, it is not entirely natural. We have therefore attempted to correct for potential artifacts within the system that could mimic a growth rate-dependent response by directly measuring promoter utilization, amount of lacZ mRNA, and plasmid copy number and by using a mycobacterial host rather than E. coli. The conclusions derived from the experiments confirm that the promoter fusion assay was a valid means of assessing GRDC of M. tuberculosis rrn promoters.

Faithful recognition and growth rate-dependent usage of M. tuberculosis rrn promoters in M. smegmatis. The utilization of the M. tuberculosis rrn P1 and P3 promoters was assessed by primer extension experiments with M. smegmatis cells carrying rrn-lacZ fusion constructs (right and wrong orientations and vector alone) grown in different media to achieve a range of growth rates. With this technique, which uses an excess of end-labelled primer to generate cDNAs complementary to RNA, the strength of the signal from the extended product obtained is a reflection of the concentration of that particular RNA species within the cell at the time of harvesting. Comparison of the signal strengths enables one to evaluate the strengths of multiple promoters for a single operon such as the rrn operon of M. tuberculosis. Primers T4In5 and T4In3 were used to assess transcription from the P1 and P3 promoters. Primer T4In5 had little sequence homology to either rrn operon of M. smegmatis and was specific for the rrn-lacZ transcript generated from plasmid construct pSD5B.16SR (Fig. 1B and C). This primer enabled the detection of two major products, tsp a and tsp c, corresponding to the P1 and P3 promoters, respectively, and two minor products (Fig. 4). The major products comigrated with RNA products a and c obtained with genomic RNA of M. tuberculosis H37Rv and H37Ra (Fig. 4B). The upstream tsp (primer extension product of ~190 bases) was directed by the P1 promoter while the tsp c (primer extension product of ~110 bases) was directed by the P3 promoter, as demonstrated previously (21, 37). The P3 tsp was preceded by the E. coli σ^70–10 and −35 consensus motifs TATTAG and TGTGACT, respectively (Fig. 1B); the sequence, position, and spacing between them closely resembled those of the P2 promoter of rrn operons from E. coli and B. subtilis. In contrast, the putative P1 promoter was composed of an E. coli σ^70–like −10 motif but lacked a −35 sequence (Fig. 1B) and resembled the bulk of M. tuberculosis promoters (4). In M. tuberculosis as well as in M. smegmatis, the signal from tsp a was weaker than that from tsp c, suggesting that the P3 promoter was the better utilized of the two promoters (Fig. 4). Transcript d, a product of RNase III-mediated processing, was generated only with genomic RNA of M. tuberculosis (Fig. 4B, lane 6), consistent with the requirement of a panhandle structure between sequences flanking 16S rRNA. From the above experiments, it was clear that (i) the rrn promoter is very strong and thus distinct from the majority of M. tuberculosis promoters and (ii) the rrn promoter is faithfully expressed in M. smegmatis.

To study rrn promoter usage in cultures growing at different rates, RNA was isolated from M. smegmatis cultures, harboring the rrn-lacZ promoter construct, grown in YK medium supplemented with 0.01 to 1% glycerol as described in Materials and Methods. Primer extension experiments were performed with primer T4In5; a five- to sixfold increase in signal intensity at a growth rate of ~1000-fold was observed with tsp c and a, respectively, over a growth rate range of 0.153 and 0.274, which correspond to cell doubling times of 6.49 and 3.64 h respectively. Further, at any given growth rate, signal c was twice as intense as signal a, suggesting that the P3 promoter is utilized more efficiently than the upstream P1 promoter (Fig. 4D) is. A second primer, T4In3, which mapped only ~55 bases downstream from tsp a (Fig. 4A), was also used, since somewhat faint signals were obtained with primer T4In5. Using T4In3, an 80-base product was generated that comigrated with the prod-
uct generated with RNA of *M. tuberculosis* (Fig. 4C). Over a growth rate range of 0.153 to 0.274, a ~12-fold increase in P1 promoter activity was noted with the T4In3 primer; the repression of P1 promoter was particularly marked below μ = 0.234 (Fig. 4C and D) and most probably represented the basal activity of this promoter. This apparent discrepancy in the degree of induction of the P1 promoter (12-fold with T4In3 versus 5- to 6-fold with T4In5) could be ascribed to variations in the efficiency of the primers to read across the rRNA sequence to yield primer extension products of significantly different lengths (191 bases for the T4In5-derived product and 80 bases for the T4In3-derived product). These primer extension studies revealed that (i) the P1 and P3 promoters of *M. tuberculosis* when in tandem are both under GRDC and (ii) the downstream promoter, which is conventionally considered to be a weak promoter in *rrn* operons in other bacteria such as *E. coli*, is well expressed in *M. smegmatis*; in fact, it is more efficiently expressed than the P1 promoter and is the major promoter.

**DISCUSSION**

*M. tuberculosis* in vivo would most probably be limited for oxygen and nutrients and therefore would display rather long generation times. However, in laboratory-grown cultures, these constraints do not exist and yet *M. tuberculosis* divides every ~18 h on average and *M. smegmatis* divides every 3 to 5 h. It is reasonable to think that multiple properties of the tubercle bacillus contribute to its slow growth. First, the unique composition of the mycobacterial envelope is likely to present permeability barriers. Since cell wall lipids constitute a high proportion of the dry weight of mycobacteria (6) and cell wall synthesis imposes a considerable energy demand on the cell, its biosynthetic rate may also be limiting for growth. Second, RNA chain growth in *M. tuberculosis* was ~10 times lower than that in *E. coli*, and the low transcription rate was attributed to a low rate of transcription initiation; rates for *M. smegmatis* were not determined (25). This was reflected in a rather low content of RNA per unit of DNA compared to that in other bacteria. Thus, in *M. bovis* and *M. tuberculosis*, the RNA/DNA ratio varied between only 1:1 and 2:1, while in *M. smegmatis*, it reached 5:1 in rapidly growing cultures (38). The G+C content of mycobacterial DNA, particularly that of the promoter regions, has been suggested as another constraint for the low rate of transcription; the upstream regions of mycobacterial genes have a higher G+C content than do the corresponding regions from *M. smegmatis* (4). rRNA gene dosage is also considered a critical factor influencing growth. Slow growers such as *M. tuberculosis* depend entirely for their total ribosome pool on a single *rrn* operon driven by two promoters, while fast growers including *M. smegmatis* typically have two *rrn* operons per genome (5) and possess multiple promoters to increase their capacity for rRNA synthesis (22).
We have demonstrated the exceptional strength of the *M. tuberculosis* *rrn* promoter in comparison with the bulk of *M. tuberculosis* promoters. We addressed whether the *M. tuberculosis* *rrn* promoter has some unique sequence or structure which precludes its modulation in conditions of varying nutrient supply. *M. smegmatis* was chosen as a surrogate host for this purpose since (i) unlike *M. tuberculosis*, it is equipped to regulate its RNA synthesis rates in response to nutrient supply (38) and (ii) it would provide a milieu devoid of the constraints impeding the growth of *M. tuberculosis*. *M. smegmatis* has been
proposed to be a good surrogate host for the study of *M. tuberculosis* transcriptional activity, protein expression, and some aspects of genetics. This is because the efficiency and fidelity of transcriptional recognition, at least for vegetative promoters, is conserved in *M. tuberculosis* and *M. smegmatis* (4). In the present study, the usage of *M. tuberculosis* *rrn* promoters was determined by primer extension analysis with genome-derived RNA from logarithmic-phase cultures of *M. tuberculosis* and comparing the signals with those obtained with plasmid-derived transcripts from *M. smegmatis* cultures harboring the *rrn-lacZ* construct. The results indicate that fidelity of transcription initiation and usage of *M. tuberculosis* *rrn* P1 and P3 promoters was maintained in *M. smegmatis*. The P3 promoter was by far the stronger *rrn* promoter in *M. tuberculosis* as well as in *M. smegmatis*. The *G+C* content of the promoter region mapping from +1 to −50 relative to the tsp showed an inverse correlation with promoter strength; it was 50 and 58% for the P3 and P1 promoters, respectively (Fig. 1), substantiating the observation that the high *G+C* content of mycobacterial promoters may have a bearing on their lower activity (4).

A surprising finding was that both the P1 and P3 promoters of the *M. tuberculosis* *rrn* operon were under GRDC. In contrast, in *E. coli* and *B. subtilis*, only one of the two promoters of intact *rrn* operons is under GRDC (12, 15), although promoter dissection experiments have demonstrated that the downstream P2 promoter also is regulatable by growth rate in *E. coli*, albeit to a lower extent than P1 (17, 20). Thus, the features in the DNA sequence that govern GRDC of the *M. tuberculosis* *rrn* promoter appear to be present around both promoters. AT-rich and upstream activating elements, Fis-binding sites, and GC discriminator sequences are characteristic features of *E. coli* *rrn* operons (12, 27, 30, 31, 33) and are involved in their regulation. Since these sequences and the Fis-encoding gene were not present in the *M. tuberculosis* *rrn* promoter or genome (11), some unique mechanism(s) is suggested for regulation. The upstream regions of *rrn* operons in *M. smegmatis* and *M. tuberculosis* are predicted to form similar secondary structures to generate potential binding sites for putative trans-acting proteins (21). It is possible that these putative trans-acting factors that participate in recognition of the structure and sequence of the *rrn* promoter regions are present in *M. smegmatis* but not in *M. tuberculosis*. A noteworthy observation was that the two promoters exhibited differential usage; the P3 promoter was ca. twice as active as the P1 promoter at all growth rates. The purpose of a weak upstream P1 promoter that is poorly expressed at all growth rates remains a puzzle. It is functionally equivalent to the downstream P2 promoter of *E. coli* *rrn* operons in relation to low level constitutive expression. Since *M. tuberculosis* possesses only one *rrn* operon, a possible advantage of having a stronger downstream promoter than upstream promoter is that promoter occlusion effects would be minimized and rRNA transcription would be maximized. On the other hand, in *E. coli*, which possesses seven *rrn* operons, the downstream P2 promoter is subject to occlusion by transcription from the P1 promoter (17).

In the context of differential usage, it may be noted that the experiments described in this report were performed with *M. smegmatis* cultures grown at various growth rates. The *M. smegmatis* cultures grown at μ = 0.234 were in logarithmic phase, while those cultured in media containing limiting amounts of glycerol, i.e., 0.05 and 0.01% glycerol (μ ≤ 0.20), reached stationary phase at 20 h of incubation, the time point at which the experiments described in this study were performed (Fig. 2). While our manuscript was in review, Gonzalez-y-Merchand et al. reported that the rate of rRNA transcription initiation from either the P1 or the P3 promoter varied little regardless of whether *M. tuberculosis* cultures were in the logarithmic or stationary phase of growth (23). These findings confirmed the observation made nearly three decades ago that RNA-DNA ratios of *M. tuberculosis* cultures altered only marginally as a function of growth rate (38). A recent report stated that in stationary-phase *M. tuberculosis* cells, the P1 promoter rather than the P3 promoter assumes charge of *rrn* transcription in a SigF-dependent manner, suggesting that sigma factor-dependent regulation of the *rrn* operon occurs in *M. tuberculosis* (16).

Despite the caveats in studying the regulation of mycobacterial genes from slow growers in rapid growers, these cross-species experiments have clearly shown that the P1 and P3 promoters of the *rrn* operon of *M. tuberculosis* are both regulatable over a range of growth rates in the environment provided by *M. smegmatis*. In conclusion, the present study clearly indicates that *rrn* promoter sequence and structure do not play a significant role in determining the low levels of RNA in *M. tuberculosis*. Other factors such as replication, cell division, cell wall biosynthesis and/or permeability, dosage of RNA genes, and absence of *trans*-acting proteins, probably serve as primary factors in determining the inability of the tubercle bacillus to respond to changes in nutrient supply.

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