The rRNA operons of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*: comparison of promoter elements and of neighbouring upstream genes

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**INTRODUCTION**

The two major mycobacterial pathogens, *Mycobacterium tuberculosis* and *Mycobacterium leprae* are characterized by the ability to grow slowly within host cells. The mechanisms which these use to control their growth rate are not well understood. There is a clear relationship between rates of growth, protein biosynthesis and the rate of cell proliferation. In addition, by comparing the growing mycobacteria would further our understanding of how these organisms use to control their growth and regulation of ribosome synthesis in the pathogenic slow-growing species with that in fast-growing mycobacteria, most of which are non-pathogenic, we are likely to gain new insights into the evolution of growth-rate control in these two phylogenetically distinct groups.

*M. leprae* and *M. tuberculosis* each have a single rRNA (*rrn*) operon (Liesack *et al.*, 1990; Sela & Clark-Curtiss, 1991; Kempsell *et al.*, 1992; Suzuki *et al.*, 1988). The single *rrn* operons of the slow-growers studied are sufficiently closely related in both their primary and secondary structures to be regarded as members of a family, described as *rrnAs* operons (the subscript ‘s’ denotes slow-growers) by Ji *et al.* (1994c). In contrast to slow-growers, fast-growing mycobacteria have two *rrn* operons (Bercovier *et al.*, 1986). The two *rrn* operons of a representative fast-grower, *Mycobacterium smegmatis*, were investigated previously (Ji *et al.*, 1994c). Based on sequence analysis, one of the operons was found to be

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Abbreviation: UNAcGCT, UDP-N-acetylglucosamine 1-carboxyvinyltransferase.

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closely related to the \( rRNA_4 \) operon of slow-growers and was designated \( rRNA_4 \) (the subscript '4' denotes fast-growers). The other \( M.\ smegmatis \) operon was designated \( rRNA_5 \) (Ji et al., 1994c); this operon appeared to be more distantly related to the \( rRNA_4 \) operon of slow-growers.

We have now cloned and sequenced the regions of the \( rRNA_4 \) and \( rRNA_5 \) operons of \( M.\ smegmatis \) upstream from the putative antitermination element, Box \( A_L \), which is highly conserved between all of the mycobacterial \( rRNA \) operons (Ji et al., 1994c). We find that the \( rRNA_4 \) and the \( rRNA_5 \) operons have similar sequences in this region, confirming our earlier hypothesis concerning the relatedness of these two operons. In addition, we have used RNA extracted from both \( M.\ smegmatis \) and \( M.\ tuberculosis \) to compare the promoter regions of the two species using primer extension. The RNA fraction of \( M.\ smegmatis \) revealed at least four transcription start points; the \( rRNA_5 \) operon appears to have a single promoter, while the \( rRNA_4 \) operon has at least three. The single \( M.\ tuberculosis \) \( rRNA_5 \) operon appears to have two promoter elements. Thus the slow-growing mycobacteria not only have a reduced number of \( rRNA \) operons, but also have a reduced number of regulatory elements involved in their transcription. Intriguingly, we also found that both \( rRNA_4 \) and \( rRNA_5 \) have a promoter, P1, which is located within a gene, which terminates 427 bp upstream of the 16S rRNA coding region. This gene encodes a protein that is significantly similar to an enzyme involved in bacterial cell wall synthesis suggesting that the production of the enzyme and transcription of the rRNA are linked.

**METHODS**

**Materials.** Restriction endonucleases and T4 ligase were from Boehringer Mannheim. Sequenase (USB) sequencing kit was supplied by Cambridge Biosciences. \(^{35}\text{S}\)dATP was from Amersham. GeneClean kit was from Bio101. The original TA cloning kit for PCR cloning was purchased from Invitrogen. The avian myeloblastosis virus (AMV) reverse transcriptase
primer-extension system was supplied by Promega. Oligonucleotides primers were prepared with an automated DNA synthesizer (model 370A, Applied Biosystems).

**Bacterial strains, media and vector.** *M. smegmatis* NCTC 8159 (National Collection of Type Cultures) was maintained on London, UK.

**Isolation of DNA.** Plasmid DNA was isolated by standard methods exactly as described previously by Ji et al. (1994c). Genomic DNA from *M. smegmatis* was isolated by a novel method (Gonzalez-y-Merchand et al., 1996). Briefly, the cells were suspended in 6 M guanidinium chloride and then kept at 65°C for 10 min. The DNA was then precipitated and resuspended in TE buffer by conventional means.

**Isolation of RNA.** Exponential-phase cells (100 ml culture) were collected and resuspended in 1 ml guanidinium buffer (6 M guanidinium chloride, 0.1% v/v, Tween 80, 10 mM EDTA, 1 mM 2-mercaptoethanol) and left at −20°C for 15 min. The suspension was added to a half of the volume of heat-sterilized 0.15 mm glass beads contained in a 2 ml screw-cap microcentrifuge tube. Mycobacteria were ruptured by 3 pulses each of 1 min, on the Mini-BeadBeater device (BioSpec Products). Debris and beads were sedimented by centrifugation (10000 g for 3 min), and the cleared lysate was retained. The pellet of beads and mycobacterial residues was briefly re-extracted on the BeadBeater (30 s pulse) with 300 μl fresh guanidinium buffer.
and the resulting extract was pooled with the first. The lysate was extracted three times with 2 vols chloroform/3-methyl-1-butanol (24:1, v/v). RNA was precipitated by the dropwise addition of 0.5 vol. ethanol and then redissolved in the appropriate volume of MOPS buffer.

**Primer extension.** The oligonucleotide primer 5’CACACTAT-TGAGTTCTC3’ has a target site which is present in all three of the *rrn* operons studied (see Fig. 2). This primer was end-labelled with [γ-32P]ATP by means of T4 polynucleotide kinase, and the primer extension was carried out using the AMV reverse transcriptase primer-extension system. Briefly, the 32P-labelled primer (100 fmol) was added to a mixture of 5 μl water containing 24 μg total RNA and 5 μl AMV primer extension (PE) 2 × buffer (100 mM Tris, 100 mM KCl, 20 mM MgCl2, 20 mM DTT, 2 mM each dNTP, 1 mM spermidine). The mixture was annealed at 52 °C for 30 min and left to cool in air at room temperature for 10 min; then 5 μl PE 2 × buffer, 1.4 μl 40 mM sodium pyrophosphate, and 1 unit AMV reverse transcriptase were added to each reaction. The reaction mixture, with ethidium bromide added at 42 °C for 1 h, extracted once with phenol/chloroform, ethanol-precipitated, and washed with 70 % (v/v) ethanol. The extension products were separated on a 8 % (w/v) polyacrylamide/urea gel and visualized by autoradiography.

**Genomic DNA cloning.** The products of the digestion with *PstI*, or double digestion with *BamHI* and *PstI* restriction endonucleases, of genomic DNA were separated by 1 % (w/v) agarose gel electrophoresis. A 32-kbp *PstI* or 1.2-kbp *BamHI/PstI* fraction was recovered from the gel using glass milk (Vogelstein & Gillespie, 1979). The *BamHI/PstI* fraction was ligated into pSK (which was previously digested with *PstI* and *BamHI*). The *PstI* fraction was ligated into pSK previously digested with *PstI*. In each case the molar ratio of vector to insert was 1:1.

**PCR.** Bacterial DNA (1–100 ng) was subjected to PCR (Saiki et al., 1988) as described previously (Ji et al., 1994c). The relevant gene fragment encoding 114 amino acids of UDP-N-acetylgalactosamine 1-carboxyvinyl-transferase (UNAcGCT) (EC 2.5.1.7), the promoter region of the *rrnA*, and *rrnA* operons, the 5’-ends of the leader regions of their precursor-RNA (pre-rRNA) and the 5’-region of the 16S ribosomal RNA was synthesized using the primer combination of JG7 (5’TGGCAAGCCAAGTATCCTTGG3’) and RAC8 (5’CAGCTGTGCTCCCGTAGG3’). The target for primer JG7 is the sequence complementary to positions 1–14 of M. *tuberculosis* (Fig. 2). The target for RAC8 is positions 339–357 of the 16S rRNA coding region (see Kempsell et al., 1992), Amplification was achieved as described previously (Ji et al., 1994c).

**PCR cloning.** The products of PCR were separated and recovered from the gel as described above. The PCR products were ligated into pCR II and transformed into One Shot InvAxF™ competent cells as specified in the manual of the original TA cloning kit. The molar ratio of vector to insert was kept at 1:1.

**Sequencing of dsDNA.** DNA sequences were determined by the dideoxy chain-termination procedure using [α-35S]dATPαS, as described by Ji et al. (1994c) using appropriate primers as indicated in Fig. 1(b).

**Alignment of sequences.** Computer-aided analysis of the alignment of nucleotide sequences of the gene encoding UNAcGCT and promoter regions was achieved by means of the BLAST program (Altschul et al., 1990). The database searches were carried out using the UK Human Genome Mapping Project computing services (Rysavy et al., 1992).

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**RESULTS AND DISCUSSION**

The scope of this study is illustrated in Fig. 1. Previously, we reported aspects of both the primary and the secondary structure of the *rrn* operons and the secondary structure of their transcripts (pre-rRNA) for *M. tuberculosis* and *M. leprae* and several other slow-growing mycobacteria (Kempsell et al., 1992; Ji et al., 1994a, b). In this study we report the upstream sequence of the *M. smegmatis* *rrnA* operon and compare the promoter elements of the two *M. smegmatis* operons with those of the single *M. tuberculosis* *rrnA* operon.

**Nucleotide sequences of the upstream regions**

The strategies described in Fig. 1 were used to establish the nucleotide sequence upstream from the Box A1 motif of the *rrnA* operon. As shown in Fig. 2, 342 bp of an ORF were identified. A search of the databases (see Methods) revealed that the amino acid sequence inferred

![Fig. 3. Putative stem–loop structures in the trailer regions of transcripts of genes located upstream from mycobacterial *rrn* operons. The nucleotide positions given in the figure refer to Fig. 2. The UAG termination codon is boxed. (a) Transcript of the gene located upstream from the *rrnA* operon of *M. smegmatis*; (b) transcript of the gene located upstream from the *rrnA* operon of *M. tuberculosis*; (c) transcript of the gene located upstream from the *rrnB* operon of *M. smegmatis*. The underlined triplet corresponds in position to part (TTG) of the putative −35 box of the P1 promoter of the *rrnB* operon.](image-url)
Fig. 4. Comparison of the locations of the 5'-ends of pre-rRNA of M. smegmatis and M. tuberculosis. The 5'-ends of pre-rRNAs were located by primer extension (see Methods) using 32P-labelled primer whose target sites overlap the Box A elements (see Fig. 2). Lanes T, C, G and A are products of sequencing reactions carried out using the same primer as mentioned above and a recombinant phagemid containing an appropriate inserted rrrn sequence. The sequencing reactions for the rrrnAf and rrrnBf operons of M. smegmatis and the rrrnA operon of M. tuberculosis are shown from left to right, respectively. The transcription start (ts) sites inferred from the products, tsA(P1), etc., indicate the operon and the promoter (see Fig. 2), and the number in parentheses signifies the distance upstream from the 5'-end of the 16S rRNA gene. a and b, Products which were not correlated with promoter-consensus sequences; r, artefact of autoradiography. Lanes 1 and 3, no RNA; 2, M. smegmatis RNA (24 µg); 4, M. tuberculosis RNA (24 µg).

from this gene sequence is significantly similar to 113 residues of the carboxyterminal region of UNAcGCT; the BLAST score was \( P = 20 \times e^{-23} \) (the lower the score the more certain is the assignment). This enzyme comprises a single polypeptide of approximately 420 amino acids (see for example Ehrt & Hillen, 1994). As shown in Fig. 2 the gene lying upstream from the rrrnA operon of M. tuberculosis is homologous with the gene which is located upstream from the rrrnA operon of M. smegmatis. Examination of M. leprae sequences (Sela & Clark-Curtiss, 1991) reveals that a gene encoding UNAcGCT is also located upstream from the rrrnA operon in that species. In contrast, the gene lying upstream from the rrrnB operon of M. smegmatis is believed to encode tyrosyl-tRNA synthetase (Predich et al., 1995).

These results confirm our original hypothesis (Ji et al., 1994c) that the rrrnA and rrrnA operons are homologous. The major difference between these two operons is a deletion of 97 bp in the M. tuberculosis operon (75 bp in that of M. leprae) compared to that of M. smegmatis, as indicated in Fig. 2.

No intrinsic terminators are evident downstream from the UAG stop codons of the ORFs (Fig. 2) indicating that termination of transcription might be rho-dependent. Possible stem–loop structures that could be involved in termination are presented in Fig. 3. In each case a GC-rich stem–loop structure can be formed within 14–21 nucleotides of the UAG codon, suggesting that the trailer regions of the transcripts comprise a minimum of 14–21 nucleotides.

**Primer-extension studies**

The RNA fraction of M. smegmatis was analysed as described in Methods and the products were identified using appropriate sequencing ladders for the rrrnA and rrrnB operons. Six major products were identified (Fig. 4). Four of the products had identifiable promoter consensus sequences immediately upstream. One of these, giving a product of 160 nucleotides, is on the rrrnB operon. Predich et al. (1995) also concluded that the rrrnB operon has a single promoter, on the basis of in vitro transcription...
studies. Three other putative promoters, giving products of 230, 136 and 60 nucleotides (designated P1, P2 and P3, respectively) have no promoter consensus of 230,136 and 60 nucleotides (designated P1, P2 and P3, respectively) are on the rrn operon. Two other products, a and the more prominent b (125 and 98 nucleotides, respectively), have no promoter consensus sequences immediately upstream of them. These could represent novel mycobacterial promoter sequences; it is known that mycobacterial promoters are poorly recognized in Escherichia coli (Kieser et al., 1986) and little information is available concerning consensus sequences for mycobacterial promoters. Alternatively these products could represent processing sites for rRNA derived from either rrnA or rrnB, or both operons. The P1 promoter of the rrnA operon is located within the coding region for UNACGCT.

Primer-extension analysis of the rrnA operon of M. tuberculosis revealed two products of 134 and 57 nucleotides (Fig. 4); promoter consensus sequences could be associated with both of these transcription start points and they appear to correspond to promoters P1 and P3 of the rrnA operon of M. smegmatis (Fig. 2), providing further evidence of the relatedness of the rrnA and rrnA operons. Two start sites were proposed previously by Verma et al. (1994) for transcription of the rrnA operon of M. tuberculosis.

As with M. smegmatis, the P1 promoter of M. tuberculosis also lies within the coding region of UNACGCT. In addition the single promoter identified on the rrnB operon is located either within or very close to the transcription region of a gene for tyrosyl-tRNA synthetase (see Fig. 2). In each case, transcription of the gene upstream from the rrn operon would be expected to influence the transcription of the rrn operon from that particular promoter. Unusual locations of promoters are also found elsewhere. Two promoters for elongation factor Tu of E. coli lie within the gene for elongation factor G (Zengel & Lindahl, 1990), and a review of 139 streptomycete transcription start sites lists at least six promoters which lie within other genes (Strohl, 1992).

The intervals separating the start sites (ts) of the promoters of the rrnA operon of M. smegmatis are 94 bp between ts(P1) and ts(P2) and 76 bp between ts(P2) and ts(P3); the start sites ts(P1) and ts(P3) directed by promoters P1 and P3 of the rrnA operon of M. tuberculosis are separated by 77 bp. These intervals are comparable to distances separating the two promoters of rrn operons of Bacillus subtilis [approx. 90 bp (Ogasawara et al., 1983)] and with the intervals between the four promoters of the rrnA operon of Streptomyces coelicolor A3(2) (van Wezel et al., 1994); namely 180 bp separating P1 and P2, 91 bp separating P2 and P3, and 80 bp separating P3 and P4.

The differential usage of the three promoters of the rrnA operon of M. smegmatis and the two promoters of the rrnA operon of M. tuberculosis merits further study. Under the growth conditions used in this study the P2 promoter of the rrnA operon was more active than either the P1 or the P3 promoters. In contrast, transcription of the rrnA operon of M. tuberculosis was governed mainly by the P3 promoter, the P2 promoter being absent from this species, with the P1 promoter playing the minor role. The presence of two or more promoters per operon is a strategy which offers the possibility of increasing the versatility of the operon, for example by enabling the operon to respond over a wide range of growth conditions to the cells' need for rRNA. Another GC-rich Gram-positive bacterium, S. coelicolor A3(2), has six rrn operons and at least two of them (rrnA and rrnD) have four promoters (Baylis & Bibb, 1988; van Wezel et al., 1994).

<table>
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<tr>
<th>Species</th>
<th>Promoter</th>
<th>Nucleotide Sequence</th>
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<tr>
<td>M. sm</td>
<td>A(P1)</td>
<td>304 GTCAACCTCGGAGCCGAGATCGGAGAGCTTAAGCTGTAAGGGAAG</td>
</tr>
<tr>
<td>M. tu</td>
<td>A(P1)</td>
<td>304 GTGAGCTCGGTTCCGAGATCGGAGGCTTAAGCTGTAAGGGAAG</td>
</tr>
<tr>
<td>M. sm</td>
<td>A(P2)</td>
<td>404 GTTTGACACTCGGACGAAACTCTGATTATCCTTTATGAGTCG</td>
</tr>
<tr>
<td>M. sm</td>
<td>A(P3)</td>
<td>480 GTTTGACACTCGGACGAAACTCTGATTATCCTTTATGAGTCG</td>
</tr>
<tr>
<td>M. tu</td>
<td>A(P3)</td>
<td>382 GTTTGACACTCGGACGAAACTCTGATTATCCTTTATGAGTCG</td>
</tr>
<tr>
<td>M. sm</td>
<td>B(P1)</td>
<td>396 GTTTGACACTCGGACGAAACTCTGATTATCCTTTATGAGTCG</td>
</tr>
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Fig. 5. Comparison of promoter elements of mycobacterial rrn operons. A(P1), etc., refer to the P1 promoter of the rrnA operon, etc.; subscript numbers denote the nucleotide positions given in Fig. 2. Asterisks denote transcription start sites identified by primer extension (Fig. 4). The TAG codons at the 3'-ends of coding regions are underlined. Putative promoter elements (−35 and −10 boxes) are framed; uncertainty in the identity of −35 boxes is shown by broken lines; a, identified as a −35 box in promoters of rrn operons of B. subtilis (Ogasawara et al., 1983); b, identified as a −35 box for several Streptomyces genes (for review see Strohl, 1992).
Each of the seven rrr operons of E. coli has two promoters (Gourse et al., 1986; Condon et al., 1992).

The promoter elements identified by means of the primer-extension studies are compared in Fig. 5. Apart from the -35 boxes of the P1 promoters of the rrrA and rrrA operons the -35 and -10 motifs are very similar to those reported for promoters of rrr operons of B. subtilis (Ogasawara et al., 1983). The P1 promoters of rrrA and rrrA operons are very similar in sequence probably because they also form part of the coding region of a gene for UNAcGCT. A feature of the P2 and P3 promoters of the rrr operon and the P3 promoter of the rrrA operon is that the -10 box is preceded by the sequence 5'GTAT3'. There appear to be few features that distinguish elements used by the fast-grower from those used by the slow-grower. It should be noted however that a strongly expressed product was identified in M. smegmatis for which no consensus -35 or -10 boxes could be identified; whether this represents a novel promoter sequence or an RNA processing site cannot be distinguished at present.

These studies confirm and extend our previous observations on the relationship between the rrr operons of fast- and slow-growing mycobacteria. More importantly they demonstrate that the two groups have evolved differently not only in terms of the number of operons, but also in terms of their regulation. Experiments currently in progress are designed to investigate the environmental stimuli which affect the differential usage of the multiple promoters and hence should provide novel insights into mechanisms of growth control in these organisms.

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REFERENCES


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