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

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***Mycobacterium smegmatis* has two rRNA (*rrn*) operons designated *rrnA*_s and *rrnB*_s. Appropriate restriction fragments of genomic DNA containing sequences immediately upstream from the 16S rRNA genes were cloned. We now report the nucleotide sequence of 552 bp upstream from the 5'-end of the Box A_L antitermination element of the leader region of the *rrnA*_s operon. The 5'-end of this segment of DNA was found to comprise 113 codons of an ORF encoding a protein which is significantly similar to UDP-*N*-acetylglucosamine 1-carboxyvinyl-transferase (EC 2.5.1.7), which is important to cell wall synthesis. A homologous ORF is located immediately upstream from the single *rrn* (*rrnA*_t) operons of *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Primer-extension analysis of the RNA fraction of *M. smegmatis* revealed four products which were related to transcription start points; the *rrnB*_s operon appears to have a single promoter whereas the *rrnA*_s operon has three (P1, P2 and P3). Analysis of *M. tuberculosis* RNA revealed two products corresponding to transcripts directed by promoters homologous with P1 and P3 of the *rrnA*_s of *M. smegmatis*. Thus, the promoter and upstream regions of the *rrnA*_s operon of *M. smegmatis* and the *rrnA*_t operon of *M. tuberculosis* are homologous. The presence of P2 in *M. smegmatis* and its absence from *M. tuberculosis* is attributable to insertions/deletions of 97 bp.**

Keywords: pathogenic/non-pathogenic mycobacteria, rRNA operons, promoter activities, cell wall synthesis, UDP-*N*-acetylglucosamine 1-carboxyvinyl-transferase

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 organisms use to control their growth rate are not well understood. There is a clear relationship between rates of growth, protein biosynthesis and the production of ribosomes (Bremer & Dennis, 1987; Winder & Rooney, 1970). An understanding of the way in which ribosome synthesis is regulated in the slow-growing mycobacteria would further our understanding of cell proliferation. In addition, by comparing the

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; Kempell *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 *rrnA*_s 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

Abbreviation: UNACGCT, UDP-*N*-acetylglucosamine 1-carboxyvinyl-transferase.

The EMBL accession numbers for the sequences reported in this paper are X87943 and X87944.

Fig. 1. Scope of this investigation. (a) Part of the *rrnA_f* operon of *M. smegmatis*. The nucleotide sequence downstream from the Box B_L antitermination element was reported previously (Ji *et al.*, 1994c). The nucleotide sequence of the V2 region of the 16S rRNA (for definition see Kempell *et al.*, 1992) serves as a signature sequence that is useful for identifying the species. This report concerns the nucleotide sequence of the region indicated by broken lines; that is, upstream from the Box A_L element to the *Pst*I site. Sequence data were obtained after cloning the *Bam*HI/(downstream) *Pst*I fragment, and after amplifying and cloning the region between the upstream *Pst*I and position 357 of the 16S rRNA coding region. The primers used for amplification are shown by short horizontal arrows. The results reveal the identity of the promoters that correspond to transcription start sites ts(P1), ts(P2) and ts(P3), and also the presence of an ORF in the position shown. (b) Sequencing strategy. Open circles indicate the binding sites of the primers used in the sequencing reaction, and the length of the arrows represent the region of sequence identified by means of the primer. (c) Part of the *rrnA_s* operon of *M. tuberculosis*. The data (see Kempell *et al.*, 1992) provide a frame of reference for (a). The segments of the operon shown in (a) and (c) are related by insertions/deletions of 97 bp in the region indicated by the open triangle (see text). ts(P1) and ts(P3) indicate the transcription start sites identified by Verma *et al.* (1994) and by primer-extension studies as described below. In both (a) and (c) the ORF corresponds to part of the gene encoding a protein significantly similar to UNAcGCT.

closely related to the *rrnA_s* operon of slow-growers and was designated *rrnA_f* (the subscript 'f' denotes fast-growers). The other *M. smegmatis* operon was designated *rrnB_f* (Ji *et al.*, 1994c); this operon appeared to be more distantly related to the *rrnA_s* operon of slow-growers.

We have now cloned and sequenced the regions of the *rrnA_f* and *rrnB_f* operons of *M. smegmatis* upstream from the putative antitermination element, Box A_L, which is highly conserved between all of the mycobacterial *rrn* operons (Ji *et al.*, 1994c). We find that the *rrnA_f* and the *rrnA_s* 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 *rrnB_f* operon appears to have a single promoter, while the *rrnA_f* operon has at least three. The single *M. tuberculosis* *rrnA_s*

operon appears to have two promoter elements. Thus the slow-growing mycobacteria not only have a reduced number of *rrn* operons, but also have a reduced number of regulatory elements involved in their transcription. Intriguingly, we also found that both *rrnA_f* and *rrnA_s* 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. [³⁵S]dATPαS 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

Fig. 2. Nucleotide sequences of the regions of the *M. smegmatis* (*M.sm*) genome upstream from the Box A_L elements of the *rrnA*_f and *rrnB*_f operons and comparison with the *rrnA*_f operon of *M. tuberculosis* (*M.tu*) [The sequence in Kempf et al. (1992) was re-determined and several errors were identified. The principal correction is the deletion of positions 73–83 from their sequence. The corrected version is given above]. The deduced amino acid sequences encoded by the putative genes for UNAcGCT (see Fig. 1) are given using the one-letter code. A single letter is given where the same amino acid is encoded by both *M.sm* and *M.tu* species, otherwise the amino acid for *M.sm* is given first; an italic letter indicates that the identity of the amino acid in the *M. smegmatis* gene is provisional because it is inferred from the sequence of the PCR primer. The *Pst*I and *Bam*HI sites are underlined and the TAG codons at the 3'-ends of coding regions are doubly underlined. Putative promoter elements (–35 and –10 boxes) are framed; uncertainty in the identity of –35 boxes is indicated by broken lines. *M.sm*(A_f), etc., *rrnA*_f operon of *M. smegmatis*, etc.; G*, transcription start points inferred from primer-extension products (see Fig. 4). tsA_f(P1), etc., transcription start site directed by the P1 promoter of the *rrnA*_f operon, etc. The sequence of the *rrnB*_f operon of *M. smegmatis* was also established (see also Fig. 4) and is in agreement with the data of Predich et al. (1995). -, Deletion; N, nucleotide of unknown identity; #, amino acid of unknown identity; dotted lines indicate where the oligonucleotide primer, used for primer-extension studies, annealed.

primer-extension system was supplied by Promega. Oligonucleotides primers were prepared with an automated DNA synthesizer (model 370A, Applied Biosystems).

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

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 - 32 P]ATP by means of T4 polynucleotide kinase, and the primer extension was carried out using the AMV reverse transcriptase primer-extension system. Briefly, the 32 P-labelled primer (100 fmol) was added to a mixture of 5 μ l water containing 24 μ g total RNA and 5 μ l AMV primer extension (PE) 2 $\times$ 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 μ l PE 2 $\times$ buffer, 1.4 μ l 40 mM sodium pyrophosphate, and 1 unit AMV reverse transcriptase were added to each reaction. The reaction mixture was incubated 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 *Pst*I, or double digestion with *Bam*HI and *Pst*I restriction endonucleases, of genomic DNA were separated by 1% (w/v) agarose gel electrophoresis. A 3.2 kbp *Pst*I or 1.2 kbp *Bam*HI/*Pst*I fraction was recovered from the gel using glass milk (Vogelstein & Gillespie, 1979). The *Bam*HI/*Pst*I fraction was ligated into pSK (which was previously digested with *Pst*I and *Bam*HI). The *Pst*I fraction was ligated into pSK previously digested with *Pst*I. 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-acetylglucosamine 1-carboxyvinyl-transferase (UNAcGCT) (EC 2.5.1.7), the promoter region of the *rrnA_t* and *rrnA_s* operons, the 5'-ends of the leader regions of their precursor-RNA (pre-rRNA) and the 5'-region of the 16S ribosomal DNA was synthesized using the primer combination of JG7 (5'-CTGCAGCCGATGGCTATCGCTTTG3') and RAC8 (5'-CACTGGTGCCTCCCGTAGG3'). The target for primer JG7 is the sequence complementary to positions 1–24 of *M. tuberculosis* (Fig. 2). The target for RAC8 is positions 339–357 of the 16S rRNA coding region (see Kempell *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 (INV α F') 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 [35 S]dATP α S, 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).

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 (Kempell *et al.*, 1992; Ji *et al.*, 1994a, b). In this study we report the upstream sequence of the *M. smegmatis* *rrnA_t* operon and compare the promoter elements of the two *M. smegmatis* operons with those of the single *M. tuberculosis* *rrnA_s* operon.

Nucleotide sequences of the upstream regions

The strategies described in Fig. 1 were used to establish the nucleotide sequence upstream from the Box A_L motif of the *rrnA_t* 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_t* operon of *M. smegmatis*; (b) transcript of the gene located upstream from the *rrnA_s* operon of *M. tuberculosis*; (c) transcript of the gene located upstream from the *rrnB_s* 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_t* operon.

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 ^{32}P -labelled primer whose target sites overlap the Box A_L 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 *rrn* sequence. The sequencing reactions for the *rrnA_f* and *rrnB_f* operons of *M. smegmatis* and the *rrnA_s* operon of *M. tuberculosis* are shown from left to right, respectively. The transcription start (ts) sites inferred from the products, $\text{tsA}_f(\text{P1})$, 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 *rrnA_s* operon of *M. tuberculosis* is homologous with the gene which is located upstream from the *rrnA_f* 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 *rrnA_s* operon in that species. In contrast, the gene lying upstream from the *rrnB_f* 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 *rrnA_f* and the *rrnA_s* 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 from 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 *rrnA_f* and *rrnB_f* 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 *rrnB_f* operon. Predich *et al.* (1995) also concluded that the *rrnB_f* operon has a single promoter, on the basis of *in vitro* transcription

Species	Promoter	Nucleotide Sequence
<i>M. sm</i>	$A_f(P1)$	304 GTCAGCCTCGGAGCCGAGATCGAGAGAGTAAGCTCGTAGGAAG* 346
<i>M. tu</i>	$A_s(P1)$	304 GTGAGTCTCGGTGCCGAGATCGAACGGGTATGCTGTTAGGCGACG* 348
<i>M. sm</i>	$A_f(P2)$	404 GATTTGCAA ^a TCGCGACGAACCTCGTATTATCTTTATGAAGTCG* 446
<i>M. sm</i>	$A_f(P3)$	480 GAC ^{a,b} TTGACAAGCCAGACAAAGCAGTATTAAGCTGGCAGGGTTG* 521
<i>M. tu</i>	$A_s(P3)$	382 GTC ^a TTGACTCCATTGCCGGATTTGTATTAGACTGGCAGGGTTG* 424
<i>M. sm</i>	$B_f(P1)$	36 GATTTGACT ^a CCCAGTTTCCAAGGACGTAACTTATTCCAGGTCA* 78

Fig. 5. Comparison of promoter elements of mycobacterial *rrn* operons. $A_f(P1)$, etc., refer to the P1 promoter of the *rrnA_f* 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).

studies. Three other putative promoters, giving products of 230, 136 and 60 nucleotides (designated P1, P2 and P3, respectively) are on the *rrnA_f* 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_f* or *rrnB_f*, or both operons. The P1 promoter of the *rrnA_f* operon is located within the coding region for UNAcGCT.

Primer-extension analysis of the *rrnA_s* 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_f* operon of *M. smegmatis* (Fig. 2), providing further evidence of the relatedness of the *rrnA_s* and *rrnA_f* operons. Two start sites were proposed previously by Verma *et al.* (1994) for transcription of the *rrnA_s* 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_f* 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_f* 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_s* 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_f* operon of *M. smegmatis* and the two promoters of the *rrnA_s* operon of *M. tuberculosis* merits further study. Under the growth conditions used in this study the P2 promoter of the *rrnA_f* operon was more active than either the P1 or the P3 promoters. In contrast, transcription of the *rrnA_s* 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).

Each of the seven *rrn* 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 *rrnA₁* and *rrnA₈* operons the -35 and -10 motifs are very similar to those reported for promoters of *rrn* operons of *B. subtilis* (Ogasawara *et al.*, 1983). The P1 promoters of *rrnA₁* and *rrnA₈* 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 *rrnA₁* operon and the P3 promoter of the *rrnA₈* 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 *rrn* 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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