The ribosomal RNA (*rrn*) operons of fast-growing mycobacteria: primary and secondary structures and their relation to *rrn* operons of pathogenic slow-growers

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The two ribosomal RNA (*rrn*) operons (*rrnA* and *rrnB*) of *Mycobacterium smegmatis* were investigated. The leader regions, part of the 16S rRNA genes, the spacer-1 regions, part of the 23S rRNA genes, and the spacer-2 regions were amplified by PCR or by inverse PCR and the products were cloned and sequenced. No differences in the sequences of the two operons were detected downstream from the Box A antitermination element of the leader region. Upstream from Box A a slow-grower-like Box B antitermination element was found in *rrnA* but not in *rrnB*. Primer extension experiments revealed that the start of transcription lies at least 370 nucleotides upstream from the 5'-end of the 16S rRNA gene and an RNase processing site near to the Box A element. Secondary structures were deduced for pre-16S rRNA and pre-23S rRNA which are distinct from, but closely related to, the corresponding structures of slow-growing mycobacteria. On the basis of these results it is proposed that the emergence of the slow-growers from the main mycobacterial line was coincident with the deletion of a segment of DNA spanning an *rrnB*-like operon, leaving an *rrnA*-like operon as the sole source of rRNA. An explanation is also proposed for the need for two Box A motifs in the transcription of an *rrn* operon based on competition between the polymerase and the nascent 30S subunit for either protein S10 and/or Box A sequences.

Keywords: fast-growing mycobacteria, slow-growing mycobacteria, precursor rRNA, Box A antitermination, rRNA processing

INTRODUCTION

Mycobacteria are Gram-positive, acid-fast bacteria. Members of the genus are conveniently classified as either slow-growers or fast-growers. These two groups are phylogenetically distinct although they are closely related (Stahl & Urbance, 1990; Rogall *et al.*, 1990; Pitulle *et al.*, 1992) based on the sequence of their ribosomal RNA. The pathogenic mycobacteria (mainly *Mycobacterium tuberculosis* and *Mycobacterium leprae*, but also including a number of species which are pathogenic to animals) are slow-growers, and it is thought that slow growth is an important attribute of their pathogenicity.

There is a broad correlation between the rate of growth of a bacterium and the number of ribosomes it produces (Bremer & Dennis, 1987; Winder & Rooney, 1970). The number of ribosomes is determined by the production of rRNA, which in turn depends on the number of rRNA (*rrn*) operons, the strength of their promoters and the efficiency with which the operons are transcribed.

It has already been demonstrated that fast-growing mycobacteria such as *Mycobacterium phlei* and *Mycobacterium smegmatis* have two *rrn* operons (Bercovier *et al.*, 1986; see also Cox & Katoh, 1986) while slow-growers have a single *rrn* operon (Bercovier *et al.*, 1986). The single *rrn* operon of the slow-growers has a classical structure (Liesack *et al.*, 1990, 1991; Sela & Clark-Curtiss, 1991; Suzuki *et al.*, 1988; Kempf *et al.*, 1992), shown in Fig. 1. The operon is transcribed as a single unit (precursor- or pre-rRNA) which is cleaved by RNases to precursors of 16S rRNA (pre-16S rRNA), 23S rRNA

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(pre-23S rRNA) and 5S rRNA (pre-5S-rRNA). Mature rRNA species are derived from the precursors by further RNase treatment.

We have previously characterized the promoter, processing sites and antitermination elements of the *rrn* operon of slow-growers (Kempsell *et al.*, 1992; Ji *et al.*, 1994a, b). In order to gain further understanding of how the slow-growing and fast-growing species control ribosome synthesis we have now further characterized the two *rrn* operons of the fast-grower *M. smegmatis*.

METHODS

Materials. Restriction endonucleases were from Boehringer Mannheim, and AMV reverse transcriptase from Life Technologies. Polynucleotide kinase and T₄ ligase were supplied by Pharmacia. Sequenase (USB) sequencing kit was from Cambridge Biosciences. [³⁵S]dATP- α S and [³²P]ATP were from Amersham. GeneClean kit was from Bio101. AmpliTaq DNA polymerase was supplied by Perkin Elmer Cetus. Oligonucleotide primers were prepared with an automated DNA synthesizer (model 370A, Applied Biosystems).

Bacterial strains and vectors. *Mycobacterium phlei* NCTC 8151, *M. smegmatis* NCTC 8159 (National Collection of Type Cultures) and *M. smegmatis* mc²155 (a generous gift from W. Jacobs, Albert Einstein College of Medicine, New York, USA; see Snapper *et al.*, 1990) were maintained on slopes of Lowenstein-Jensen medium and stored at room temperature. pBluescript (pKS) vector (Stratagene) was used for cloning mycobacterial DNA; the host was *Escherichia coli* strain DH5 α F'. The shuttle vector pEJ106 was given by Dr E. Davis, National Institute for Medical Research, Mill Hill, London; it was used to clone mycobacterial DNA first into *E. coli* and then into *M. smegmatis* mc²155.

Media. *M. phlei* and *M. smegmatis* were grown overnight at 37 °C in 5 ml Lemco broth containing 0.05% Tween 80. This culture was used to inoculate 200 ml of the Lemco Tween 80 medium. Large cultures were incubated at 37 °C with rotary shaking (150 r.p.m.; model G25 Shaking Incubator, New Brunswick Scientific). *M. smegmatis* for electroporation was grown in modified Dubos liquid medium (Dubos & Davis, 1946) for 24 h, as described above. After electroporation *M. smegmatis* was plated on Middlebrook 7H10 agar (Difco) containing 10% (v/v) Dubos medium albumin supplement (Difco) and 10–15 μ g kanamycin ml⁻¹. Purity of cultures was checked using acid-fast staining.

E. coli was grown in Luria-Bertani (LB) or 2 $\times$ TY medium, with antibiotics added as required (Sambrook *et al.*, 1989). Cultures were incubated at 37 °C (200–330 r.p.m.) for 18–24 h.

Construction of plasmid pJY104. The 1.2 kbp *Pst*I fragment of the *rrn* operon of *M. tuberculosis*, which contains nearly 650 bp of 16S rRNA gene coding region and 550 bp of its 5'-end flanking region, was introduced into the multiple cloning site of the vector pEJ106, which contains a chloramphenicol acetyltransferase (*cat*) gene lacking its own promoter. The pJY104 construct contains the promoter in the correct orientation for the expression of the *cat* gene (Ji *et al.*, 1994a). The construct was used to transform both *E. coli* DH5 α F' and *M. smegmatis* mc²155. After transformation, the rRNA was isolated.

Transformation of *M. smegmatis* by electroporation. Electroporation of mycobacteria was carried out using an adaptation of the method described by Snapper *et al.* (1990). One millilitre of an overnight culture of *M. smegmatis* mc²155 was used to inoculate 100 ml modified Dubos medium and the

culture was grown at 37 °C for 24 h. The cells were harvested by centrifugation (220 g for 10 min), washed in 10% (v/v) glycerol and resuspended in 10 ml 10% (v/v) glycerol.

Plasmid DNA, up to 1 μ g, was added to 0.8 ml of the cell suspension and placed in an electroporation cuvette (0.2 cm electrode gap) on ice for 10 min. The cells were then given a single pulse of 25 μ F at 6250 V cm⁻¹ with resistance set at ' α ' (Gene Pulser Apparatus, Bio-Rad) and replaced on ice for 10 min. An equal volume of Lemco broth was added and the cells incubated at 37 °C for 2 h. Cells were pelleted in a microcentrifuge at 1300 r.p.m. for 5 min, resuspended in 100 μ l Lemco broth and plated on 7H10 agar plates containing 15 μ g kanamycin ml⁻¹.

Isolation of DNA. Plasmid DNA was isolated by standard methods exactly as described previously (Ji *et al.*, 1994a). Genomic DNA was isolated from mycobacteria by the method of Katoch & Cox (1986), avoiding the use of the French pressure cell (see Ji *et al.*, 1994a). RNA was hydrolysed by incubation with 20000 units of RNase T1 (Life Technologies) overnight at 37 °C in TE buffer. The RNase was removed by phenol/chloroform extractions and the DNA was then ethanol-precipitated (using 0.1 vol. 3 M sodium acetate and 2 vols ethanol) and finally dissolved in 100 μ l TE buffer. The yield was 2.7 mg DNA from 7.2 g (wet weight) of *M. smegmatis*.

Isolation of RNA. *M. smegmatis* NCTC 8159 or mc²155 was harvested by centrifugation, resuspended in lysis buffer and passed through a French pressure cell at 18000 p.s.i. (124 MPa) at -10 °C (see Katoch & Cox, 1986). Insoluble debris was removed by centrifugation at 5000 g for 10 min and the supernatant was deproteinized by repeated extraction with chloroform/3-methyl-1-butanol (24:1, v/v). RNA was precipitated by the dropwise addition of 0.5 vol. ethanol.

Primer extension. The oligonucleotide primers 5'-CAC ACT ATT GAG TTC TC-3', which is complementary to positions 46–62 (see Fig. 2) of the leader region, and 5'-CTG AGC CAG GAT CAA ACT CTCC-3', complementary to positions 208–229 (Fig. 2) (positions 6–27 of the 16S rRNA coding region) were end-labelled with ³²P by means of T4 polynucleotide kinase, then gel-purified and dissolved in double-distilled water. The ³²P-labelled primer (10 ng) was added to 7 μ l water containing 10–30 μ g total RNA. The mixture was denatured at 97 °C for 10 min and 2 μ l of 2 $\times$ annealing buffer (50 mM PIPES pH 6.4, 2 M NaCl) was added. The nucleic acids were annealed at 52 °C for 20 min, then 90 μ l reverse transcriptase buffer (60 mM NaCl, 10 mM NaCl, 10 mM Tris/HCl pH 8.3, 10 mM DTT, 8 mM MgCl₂, 1 mM each of dCTP, dGTP, dATP and dTTP, and 50 μ g actinomycin D ml⁻¹) containing 100 units ml⁻¹ avian myeloblastosis virus (AMV) reverse transcriptase was added to each reaction. The reaction mixture was incubated at 42 °C for 1 h, extracted twice with phenol/chloroform, ethanol-precipitated, and washed twice with 70% (v/v) ethanol. The extension products were separated on a 12% (w/v) polyacrylamide-urea gel and visualized by autoradiography.

DNA polymerase chain reaction (PCR). Bacterial DNA (1–100 ng) was subjected to PCR in a total volume of 50 μ l, with 1 unit *Taq* polymerase, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatin, 100 pmol of each of two appropriate primers and 200 μ M each of dATP, dCTP, dGTP and dTTP. The 50 μ l mixture was covered by 50 μ l of light mineral oil. The relevant gene fragment coding for the leader region was synthesized using primer combination 1 (primer L1 5'-GGG TTG CCC CGA AGC G-3'; and cg8 5'-CAC TGC TGC CTC CCG TAG G-3'; see Ji *et al.*, 1994b) Spacer-1 was amplified using primer combination 2 (g5 5'-GCC AAG GCA TCC ACC ATG C-3'; and cg10 5'-ATT GAC GGG GGC CCG CAC AAG CG-3') as described by Ji *et al.* (1994b); and the gene

fragment coding for spacer-2 was amplified using primer combination 3 (S2-1, 5'-CCA GTT CTC TCA CCA GGG G-3'; and S2-2, 5'-CAC ACA CAT CGC AAC CAC-3'). The target for primer S2-1 is a sequence located approximately 210 bp upstream from the 3'-end of the 23S rRNA gene of *M. leprae* (positions 3224–3242, EMBL Data Library Accession Number X56657; Liesack *et al.*, 1991). The target for primer S2-2 is positions 42–59 of the 5S rRNA gene (see Dams *et al.*, 1987). Amplification was achieved using 36 cycles. The reaction mixture was heated to 90 °C for 1 min, kept at 58 °C for 1 min, then heated to 70 °C for 2.5 min, and this cycle was repeated 35 more times. Finally, the solution was heated to 94 °C for 15 s, then kept at 58 °C for 1 min and at 72 °C for 5 min.

Inverse PCR (Triglia *et al.*, 1988). Genomic DNA of *M. smegmatis* NCTC 8159 was digested with *Bam*HI for 2 h at 37 °C. Circularization was performed in the same tube with T4 DNA ligase in a low DNA concentration (5 µg ml⁻¹) that favours the formation of monomeric circles. Ligase activity was destroyed by heating to 68 °C for 15 min. After phenol-extraction and ethanol-precipitation, the digested and circularized DNA was resuspended in 10 µl water. Two primers [5'-GTG CCA GCA GCC GCG GTA ATA CG-3', identical to the *M. tuberculosis* 16S rRNA gene sequence at positions 1126–1148 (Kempell *et al.*, 1992); and cg8, 5'-CAC TGC TGC CTC CCG TAG GAG T-3', complementary to the *M. tuberculosis* 16S rRNA gene sequence at positions 960–978] were used with their 3'-end facing the unknown region. The other conditions were the same as for normal PCR.

PCR cloning. The methods described by Ji *et al.* (1994b) were used. The products of PCR were separated by agarose gel electrophoresis. DNA was recovered from the gel by using glass milk (Vogelstein & Gillespie, 1979). Bluescript phagemid was digested with *Eco*RV and modified by the addition of a single thymidine at the 3'-ends to facilitate the ligation with PCR products which are found to have an overhanging adenosine residue at the 3'-end (Clark, 1988). The ratio of vector to insert was kept 1:1.

Sequencing of double-stranded DNA. At least five colonies from the same ligation reaction were selected, cultured and plasmid DNA isolated. Sequences of the inserted DNAs were determined. This procedure reduces the possible errors caused by PCR amplification (Saiki *et al.*, 1988). DNA sequences were determined by the dideoxy chain-termination procedure using [³⁵S]dATP-αS, as described by Ji *et al.* (1994a).

RESULTS

The scope of this study and the strategies employed are described in Fig. 1. Nucleotide sequence data were obtained for the leader regions of the *rrnA* and *rrnB* operons of *M. smegmatis* and for the spacer-1 and spacer-2 regions of the two operons. The sequence data are summarized in Fig. 2. Nucleotide positions are numbered from the 5'-end of the section of the leader region established for *rrnA*. The sizes of the regions which were studied are given in Table 1. The three antitermination elements, Box A, Box B and Box C, found in the leader and spacer-1 regions (Berg *et al.*, 1989), are distinguished by the subscripts L (for leader) and S (for spacer-1).

The leader region

The *rrnA* operon. Previously we identified a putative Box B_L antitermination element near to the site for the start of transcription of the *rrn* operon of *M. tuberculosis* (Kempell

et al., 1992). Later, an identical or very similar sequence was detected in all eight of the slow-growers studied (Ji *et al.*, 1994a). Genomic DNA was amplified by using one primer (L1) directed at the putative Box B_L element and another (cg8) directed at a highly conserved sequence of the 16S rRNA coding region. The products ranged in size from 539 to 563 bp, and comprised 183–207 bp of 5'-flanking sequence and approximately 365 bp of the 16S rRNA gene (Ji *et al.*, 1994a). The same two primers generated a product of 559 bp when genomic DNA of *M. smegmatis* was the substrate for the PCR reaction. The product was cloned and sequenced (see Fig. 2). On the basis of the assumption that the 5'-end of 16S rRNA is pUUG (see Fig. 2) the product comprised 202 bp of 5'-flanking sequence and 357 bp of the 16S rRNA coding region.

The part of the genome which was amplified by the above-mentioned procedure was found to be located within the 1.4 kbp *Pst*I fragment of the *rrnA* operon (see Fig. 1). Genomic DNA was restricted with *Pst*I, the products were separated according to size by 1% (w/v) agarose gel electrophoresis and DNA was recovered from the gel. The pool of DNA fragments of 1.1–1.7 kbp containing the 1.4 kbp fragment of the *rrnA* operon yielded a product with primers L1 and cg8 by PCR whereas the 2.7–3.7 kbp fraction containing the 3.2 kbp fragment of the *rrnB* operon did not (results not shown). We infer that *rrnA* has a motif very similar to the Box B_L antitermination element found in all the slow-growers studied (Ji *et al.*, 1994a) and that this motif is absent from *rrnB*.

The *rrnB* operon. The region upstream from the 5'-end of the 16S rRNA coding region of the *rrnB* operon was investigated by means of the inverse PCR procedure (see Fig. 1 and Methods). The product of 925 bp which was obtained after the final step of amplification by PCR was cloned and sequenced (see Methods). It was found to comprise 334 bp of the 16S rRNA gene (positions 695–1028, Fig. 2; positions 493–826 of the coding region), followed by 234 bp of the leader region (positions –31 to 202), followed by 357 bp of 16S rRNA gene (positions 203–559; positions 1–357 of the coding region). The results (Fig. 2) confirm the absence from *rrnB* of the Box B_L motif found in slow-growers. However, downstream from position 33 (Fig. 2) the sequences of *rrnA* and *rrnB* were identical.

The start of transcription

The target for the ³²P-labelled primer used to investigate the start of transcription was the sequence 46–62 (Fig. 2) which is present in the leader regions of *rrnA* and *rrnB* and also the *rrn* operons of slow-growers.

When the RNA fraction of *M. smegmatis* was investigated by the primer extension method, the principal product was DNA of approximately 230 nucleotides (nt), with minor products of approximately 135 nt and approximately 162 nt, which could be the result of RNase cleavage of the major product. Thus, the start of transcription of

Fig. 1. Strategies used in this investigation. (a) *rrn* operons of *M. smegmatis*. The *Bam*HI (B) and *Pst*I (P) sites serve to distinguish *rrnA* from *rrnB* (Bercovier *et al.*, 1989). The same primers which amplify the leader region of slow growers (Ji *et al.*, 1994a) also amplify part of the leader region of *rrnA* but not *rrnB*. This reveals the presence of a slow-grower-like Box B antitermination element in *rrnA* and its absence from *rrnB*. Part of the leader region of *rrnB* was obtained by first circularizing the *Bam*HI fragment and then amplifying by inverse PCR (Triglia *et al.*, 1988) as described in Methods. The primers used to amplify spacer-1 and spacer-2 amplify both *rrnA* and *rrnB*. Amplified DNA was cloned and sequenced (see Methods). Box A and Box B are antitermination elements. Helix 10, which is part of the variable region V2, serves as a signature sequence useful for identifying the species (Ji *et al.*, 1994a). $\triangleright$ — $\triangleleft$, regions of genomic DNA amplified by PCR; the arrowheads indicate the target sites for the primers. (b) Putative transcripts of *rrnA* and *rrnB* operons (approx. 5.6 kbp). The two transcripts differ in nucleotide sequence upstream from the Box A antitermination element. The location of the 5'-end of the transcripts was investigated by the primer extension method using reverse transcriptase. The target for the primer was a sequence spanning the Box A element, as shown. The broken lines denote uncertainties in the locations of the 5'- and 3'- ends of the transcripts. $\blacktriangleleft$ — $\bullet$, products of primer extension experiments: the filled circle represents the primer and the arrow denotes the direction of synthesis of the product. (c) Processing of pre-rRNA yielding intermediate products. The interactions between leader and spacer-1a sequences in pre-16S rRNA and between spacer-1b and spacer-2 in pre-23S rRNA are represented by dots. In (a–c) the hatched bars represent coding regions of the operons and their transcripts.

both *rrnA* and *rrnB* is at least 370 bp upstream from the 5'-end of the 16S rRNA gene, and at least 170 nt from the 5' end of the Box B_L element.

To provide a frame of reference, the site for the start of transcription of the *rrn* operon of *M. tuberculosis* was investigated in *M. smegmatis* transformed with the promoterless plasmid pJY104. A new primer extension product of 56 nt was obtained (see Fig. 3a). A product of

identical size was obtained previously when *E. coli* was transformed with plasmid pJY104 (Ji *et al.*, 1994a). The start of transcription of the *rrn* operon of *M. tuberculosis* is approximately 200 bp upstream from the 5'-end of the 16S rRNA gene, close to the 5'-end of the Box B element corresponding to position 3 (Fig. 2). In contrast, transcription of the *rrnA* and *rrnB* operons of *M. smegmatis* starts at least 170 bp further upstream (see Fig. 3a and Table 1). Thus, neither *rrnA* nor *rrnB* has a site for the

Table 1. Sizes of the leader, spacer-1 and spacer-2 regions of the *rrnA* operon of *M. smegmatis* and comparison with *M. leprae*

Data for *M. leprae* are from Liesack *et al.* (1990, 1991), Sela & Clark-Curtiss (1991) and Kempell *et al.* (1992).

Region	Size (bp)		Difference in size (bp)* [<i>M. sm.</i> – <i>M. le.</i>]	Location of size differences	Explanation of size difference
	<i>M. sm.</i>	<i>M. le.</i>			
Leader					
Complete	≥ 370	205	≥ 165	See below	See below
5'-end to Box B _L	≥ 170	4	≥ 166	Upstream from Box B _L	Difference in the location of promoters
Box B _L to 3'-end	200	201	1	Not applicable	Not applicable
Spacer-1					
Complete	362	282	+ 80	See below	See below
Spacer-1a	162	129	+ 33	1830–1864	Expanded helix S1-4
Spacer-1b	200	153	+ 47	1912–1933 1998–2069	Box B _s ^{'''} (extra stem-loop) Expanded Helix S-5
Spacer-2					
Complete	102	85	+ 17	See below	See below
Spacer-2a	52	63	– 11	5214–5224	Uninterrupted c(stem-S1)
Spacer-2b	50	22	+ 28	5265–5291	Helix S2-2 (extra stem-loop)

* The sizes are based on the 5'- and 3'-ends of 16S rRNA and 23S rRNA indicated in Fig. 4 and in Ji *et al.* (1994a, b).

identified in the leader region of the *rrnO* operon of *Bacillus subtilis* (Ogaswara *et al.*, 1983).

A putative RNase III site was identified in spacer-1b [approximate position 1971 (S1-244), Fig. 2] on the basis of sequence similarities with known sites (cf. Ji *et al.*, 1994b).

The 16S rRNA coding region

Several parts of the 16S rRNA gene were amplified by PCR, cloned and sequenced. Two 5'-fragments (positions 1–357 of the mature 16S rRNA sequence) were derived independently from *rrnA* and *rrnB*. The same nucleotide sequence was obtained for each fragment. Part of this sequence, the variable region V2 (positions 138–220 of the 16S rRNA gene), is presented in Fig. 2. This region, especially helix 10, is a valuable guide to the identity of a particular species.

The two other parts of the 16S rRNA gene investigated were positions 493–832 of the *rrnB* operon and positions 899–1526 of *rrnA* and *rrnB*. Partial sequences were obtained and were compared with the data of Rogall *et al.* (1990) for positions 40–1493 of the 16S rRNA gene (EMBL Data Bank accession number X52927). Where comparison is possible the two sets of data are in agreement.

The combined data of Rogall *et al.* (1990) and ourselves suggest an overall length of 1526 nt for the 16S rRNA gene on the basis of the assumption that the 5'-end is pTTTG (see Fig. 2). The 5'-end of *M. smegmatis* 16S

rRNA was not identified directly and the number of T residues at the 5'-end is in the range 3–5 (cf. the 5'-end of slow-growers; Ji *et al.*, 1994a). The assignment of position 4 to the first G residue of the 16S rRNA sequence facilitates comparison with the *M. tuberculosis* 16S rRNA gene sequence (Kempell *et al.*, 1992).

The spacer-1 and spacer-2 regions

rrnA and *rrnB* have the same overall structure (see Fig. 1) as judged by restriction mapping (Bercovier *et al.*, 1989). The binding sites of the primers used to amplify the spacer-1 and spacer-2 regions are highly conserved sequences of the 16S rRNA, 23S rRNA and 5S rRNA genes. Thus the *rrnA* and *rrnB* operons should have identical targets for the primers and should be amplified in equal proportions, and clones of *rrnA* and *rrnB* sequences should be equally abundant. All the clones examined yielded the same spacer-1 or spacer-2 sequence. We infer that the nucleotide sequences of the spacer-1 and spacer-2 regions of *rrnA* and *rrnB* are identical. The sequences of the 3'-end of the 16S rRNA gene, the spacer-1 region, the 5'- and 3'-ends of the 23S rRNA gene, spacer-2 and the 5'-end of the 5S rRNA gene are given in Fig. 2. The sizes of the spacer-1 and spacer-2 regions are summarized in Table 1.

Secondary structure of the *rrnA* operon

The leader, spacer-1, spacer-2 and trailer regions play a crucial role in determining the three-dimensional structure of pre-rRNA, from which the mature rRNA species

Fig. 4. Schemes for the secondary structure of part of the transcript (pre-rRNA_A) of the *rrnA* operon of *M. smegmatis*. (a) Scheme for the section (see Fig. 2) extending from the 5'-end of primer L1 to the 3'-end of spacer-1A (nucleotides 1–1889). The 5'-end of the transcript is at least 170 nucleotides upstream from the putative Box B_L antitermination element. (b) Scheme for the section extending from the 5'-end of spacer-1b to the 5'-end of mature 5S rRNA (nucleotides 1890–5307). The scheme, which is supported by the extensive sequence similarities, was first proposed for pre-rRNAs of slow-growing mycobacteria (Ji *et al.*, 1994a, b). Pre-rRNA_A and pre-rRNA_B (the transcript of the *rrnB* operon) are believed to be identical downstream from nucleotide 33 (L33), as shown in Fig. 2. Mature 16S rRNA, 23S rRNA and 5S rRNA sequences are boxed. The broken lines indicate uncertainty in the locations of the 5'- and 3'-ends of 16S rRNA and 23S rRNA, the approximate locations of which are shown by labelled arrows. Part of the loop of helix L1 and of helix S4 is boxed to indicate a possible interaction between the two motifs in the tertiary structure. The capacity of the 5'-terminal sequence of spacer-1 to interact with both the 5'- and 3'-ends of 16S rRNA is illustrated (see helix S1-1). The potential RNase III cleavage site, position L51, was identified by primer extension (see Fig. 3b). The putative RNase E site near S1-244 and the putative RNase E site near S2-64 were identified by means of sequence similarities (Régner & Hajsndorf, 1991). A–U, G–C indicate standard 'Watson and Crick' base-pairs; G–U, etc., indicate possible 'wobble' base-pairs.

are derived by the action of RNases. Previously, a scheme of secondary structure for pre-rRNA was proposed which accommodates the nucleotide sequences of *M. leprae*, *M.*

tuberculosis and all six other slow-growers studied (Ji *et al.*, 1994a, b). This same scheme of secondary structure applies to the *rrnA* operon of *M. smegmatis*. All the main

Fig. 5. Possible secondary structure of part of pre-rRNA_B. Pre-rRNA_B differs from pre-rRNA_A in the nucleotide sequence upstream from position 33 of *rrnA* (see Fig. 2), as shown by the lower-case letters. Downstream from position 33 both transcripts appear identical in sequence (upper-case letters) and also in secondary structure.

features described for the secondary structure of slow-growers are also found in the *rrnA* operon. However, there are differences in detail which are described in Table 1. In the pre-16S rRNA moiety the principal difference lies in the location and size of helix S1-4. In slow-growers this helix is located within approximately 5 base-pairs of helix L1 and comprises approximately 2 base-pairs. In contrast, helix S1-4 of the *rrnA* operon is more distant (15 base-pairs) from helix L1 and is stabilized by approximately 14 base-pairs, although four of them are G–U wobble base-pairs.

The slow-growers have two Box B_s stem-loop structures at the 5'-end of spacer-1b. In contrast, a third potential Box B-like motif (Box B_s^{'''}) is present in the *rrnA* operon and helix S1-5 is longer than in *M. leprae*. In pre-23S rRNA the long stem is uninterrupted (see Fig. 4) whereas in slower-growers Helix S2-1 is found near to the putative RNase III cleavage site within the pre-23S rRNA stem.

Lastly, the *rrnA* operon has an extra potential stem-loop structure located between the potential RNase E cleavage site and the 5'-end of the 5S rRNA gene. As discussed below, these differences are reflected in the size of pre-16S rRNA, pre-23S rRNA and pre-5S rRNA derived from the *rrnA* operon compared with their counterparts derived from pre-rRNA of slow-growers.

The transcript, pre-rRNA, is first cleaved by RNase III to produce pre-16S rRNA and pre-23S rRNA; and by RNase E to produce pre-5S rRNA. Cleavage at these sites would yield pre-16S rRNA comprising mature 16S rRNA extended by approximately 149 nt upstream from the 5'-end and approximately 149 nt downstream from the 3'-

end (5'-149 nt–16S rRNA–149 nt-3'). Pre-23S rRNA is likely to have the structure 5'-118 nt–23S rRNA–24 nt-3'. Cleavage at the putative RNase E site (position S2–64) would lead to pre-5S rRNA comprising mature 5S rRNA extended by 38 nt upstream from the 5'-end. The estimated sizes of the *M. leprae* precursors (Ji *et al.*, 1994a, b) are: pre-16S rRNA (5'-160 nt–16S rRNA–113 nt-3'), pre-23S rRNA (5'-94 nt–23S rRNA–27 nt-3') and pre-5S rRNA (5'-11 nt–5S rRNA–33 nt-3').

Secondary structure of the *rrnB* operon

The nucleotide sequence of the *rrnB* operon differs from that of the *rrnA* upstream from position 33 (Fig. 2). A possible secondary structure of this region of the transcript of the *rrnB* operon is given in Fig. 5. Downstream from position 33 the secondary structure of pre-rRNA_B is probably identical with that of pre-rRNA_A.

Comparison with other fast-growers

The notion that the *rrn* operons of *M. smegmatis* may be representative of fast-growers in general is supported by data obtained for the leader and spacer-1 regions of *M. phlei*. Genomic DNA of *M. phlei* is amplified by primers L1 and cg8 yielding a product of approximately 560 bp (Ji, 1993), which is the same size as the product obtained for *M. smegmatis*. The inference is that at least one of the *rrn* operons of *M. phlei* also has a slow-grower-like Box B antitermination element approximately 202 bp upstream from the 5'-end of the 16S rRNA gene.

The spacer-1 of *M. phlei* was amplified by PCR (results not shown). It was found to be comparable in size (355 bp) with the *M. smegmatis* spacer-1 region (362 bp). van der Giessen *et al.* (1994) have reported the nucleotide sequence of the spacer-1 region of *M. phlei*. The *M. phlei* and *M. smegmatis* sequences are compared in Fig. 6. The high degree of homology between the two species includes the features which distinguish *M. smegmatis* from the slow-grower, namely an enlarged (33 nt) helix S1-4 in spacer-1a (see also Fig. 4a) and an extra stem-loop (32 nt) structure (Box B_s^{'''}) in spacer-1b.

van der Giessen *et al.* (1994) also showed that each of the fast-growers *Mycobacterium fortuitum* and *Mycobacterium chelonae* has a spacer-1 region which is comparable in size with the spacer-1 region of *M. smegmatis*. Thus the sizes of the spacer-1 regions of the *rrn* operons of these fast-growers serve to distinguish them from slow-growers, as illustrated in Table 1.

Comparison with slow-growers

The *rrnA* operon. Although the *rrnA* operon of *M. smegmatis* has distinctive features it is clearly closely related to the single *rrn* operon of *M. leprae* (see Table 1). There are several regions of the *rrn* operons of slow-growers which were found to have identical sequences (Ji *et al.*, 1994a, b). The same (or very similar) sequences are also present in the *rrnA* operon. The regions of high homology are as follows: 31 bp of the leader region [positions 38–68 (L38–L68); Fig. 2]; the complementary

Fig. 6. Comparison of the primary and possible secondary structures of the spacer-1 regions of the *rrn* operons of *M. smegmatis* (*M. sm*; see Fig. 2) and *M. phlei* (*M. ph*; see van der Giessen *et al.*, 1994). Possible features of the secondary structure of the pre-rRNA transcript are indicated by the labelled arrows and short vertical lines, which mark complementary sequences and, where appropriate, the intervening hairpin loop. Stem-loop structures are numbered in sequence (helix S1, etc.) from the 5'-end of spacer-1. The sequence designated c(Stem-L) of the spacer-1a region has the capacity to interact through base-pairing with sequences of the leader region. Similarly, sequences labelled Stem-1b may interact with sequences of spacer-2, as illustrated in Fig. 4(a, b). –, Deletion; *, nucleotide common to *M. smegmatis* and *M. phlei*. 23S rRNA gene sequences are boxed; the broken line denotes uncertainty in the location of the 5'-end.

sequence found in spacer-1a [positions 1859–1889 (S1-132 to S1-162); Fig. 2]; a sequence of 44 bp in spacer-1b [positions 1944–1987 (S1-217 to S1-260); Fig. 2]; and the complementary regions located in spacer-2a [positions 5190–5199 (S2-5 to S2-14) and positions 5223–5237 (S2-38 to S2-52); Fig. 2]. In addition, there are extensive sequence homologies as shown by the scheme of secondary structure of pre-rRNA_A (see Fig. 4) and pre-rRNA of slow-growers (Ji *et al.*, 1994a, b).

Antitermination motifs. Possible antitermination elements present in the non-coding regions of the *rrnA* operon of *M. smegmatis* are also present in the *rrn* operon of *M. leprae*, which is representative of slow-growers (Ji *et al.*, 1994a, b). Elements identical to Box B_L, Box A_L and Box C_L found in *M. smegmatis* are also present in *M. leprae*. The Box B_S' and Box B_S'' of the *rrnA* operon are homologues of their counterparts in *M. leprae* but they are separated by a third stem-loop structure (Box B_S'''); the Box A_S element of the *rrnA* operon is 5'-UGGUGUUUGAUU-3' and so differs in one position from Box A_S of *M. leprae* (5'-UGGUGUUUGAGU-3') whereas Box C_S is identical for both *rrnA* and the slow-grower. These similarities suggest that the mechanisms of antitermination, particularly those involving Box A, are very similar for *M. smegmatis*, *M. leprae* and other slow-growers.

The *rrnB* operon. The mechanisms of antitermination

and of processing by means of RNase cleavages discussed above for the *rrnA* operon and its transcript pre-rRNA_A also apply to the *rrnB* and its transcript pre-rRNA_B. The two operons are identical downstream from position 33 and differ in that a slow-grower-like Box B_L element is absent from *rrnB*. The significance of this difference remains to be evaluated.

DISCUSSION

The sequence data obtained for the non-coding regions of the two *rrn* operons of the representative fast-grower *M. smegmatis* extend our knowledge of *rrn* operons of mycobacteria. These data, together with previous work (Ji *et al.*, 1994a, b), support two conclusions. First, there are features of the non-coding regions which are characteristic of the genus; these include the highly conserved sequences of the leader, spacer-1 and spacer-2 regions (see above), and the overall scheme of secondary structure proposed for pre-16S rRNA and pre-23S rRNA (see Fig. 4). Secondly, there are clear differences between fast-growers and slow-growers which are evident in the spacer-1 and spacer-2 (see Table 1).

Box A antitermination motifs

The mycobacterial Box A_L and Box A_S sequences are related to, but different from, the Box A sequences reported for other species (Berg *et al.*, 1989; Ji *et al.*,

1994a, b). One function of the Box A sequences of pre-rRNA is to form a complex with transcription factors, in particular with a heterodimer of NusB and S10 or their analogues, which may then attach to the polymerase (Nodwell & Greenblatt, 1993).

Why does each *rrn* operon have two Box A elements?

This question was considered taking into account the proposed secondary structure of pre-rRNA (Fig. 4) and on the basis of the assumption that, as transcription proceeds, the growing pre-rRNA chain folds forming stem-loop structures, etc., to which ribosomal proteins bind in a defined order. The growing transcript of the 16S rRNA gene and its associated proteins may be regarded as the precursor of the small (30S) subunit.

We propose that the two Box A elements are needed for the complete transcription of the *rrn* operon because the copy of the NusB.S10 heterodimer, which attaches to the polymerase at the Box A_L element, is detached from the polymerase at a point where transcription of the 16S rRNA gene nears completion and before the 3'-end of spacer-1a is reached. To prevent premature transcription termination of the 23S rRNA gene and 5S rRNA genes, a second copy of the NusB.S10 heterodimer attaches to the Box A_S sequence and stays with the polymerase until transcription of the operon is complete, or at least nears completion.

There are several ways in which the NusB.S10 heterodimer might be displaced from the polymerase. One possibility is based on competition for the Box A_L sequence bound to the NusB.S10 heterodimer. Helix S1-1 (Fig. 4a) brings together the 5'- and 3'-ends of 16S rRNA and so facilitates the formation of the long stem of pre-16S rRNA which is formed through the interaction of stem-L sequences of the leader region with c(stem-L) sequences of the spacer-1a region. Box A_L is part of the stem-L sequence. Hence, formation of the stem is based on competition between the c(stem-L) sequence and the NusB.S10 heterodimer for Box A_L. As a result of the formation of the stem, the NusB.S10 heterodimer is lost from the polymerase and a second copy attaches to the polymerase at Box A_S, and remains attached until the formation of the pre-23S rRNA stem. There is no Box A element present in the spacer-2 region so that the 5S rRNA gene would be transcribed in the absence of a NusB.S10 heterodimer as a component of the polymerase.

The nascent ribosomal subunit has the potential to compete with the RNA polymerase for S10. Taking the *E. coli* ribosome as a model, a single copy of S10 is located within the 3' major domain of the small subunit, where it is stabilized by interactions with its protein neighbours S3, S9 and S14 and by interaction with 16S rRNA sequences 1130–1280 (Stern *et al.*, 1989). Incorporation of S10 into the small subunit could take place after elongation of the transcript to nucleotide 1390 if, as in the assembly of the subunit *in vitro* (Nomura, 1973), incorporation of S10 follows the incorporation of protein

S7, which has a complex 16S rRNA binding site (Stern *et al.*, 1989). Thus, a second possibility is that at a point late in the transcription of 16S rRNA gene the nascent small subunit may compete with the RNA polymerase for the copy of S10 present as a heterodimer with NusB, leading to the transfer of S10 from the polymerase to the nascent subunit. NusB could be said to act as a temporary scaffold or chaperone for S10. The heterodimer which subsequently attaches to RNA polymerase at Box A_S will remain attached until transcription is complete.

A third possibility is that both competing reactions are coordinated so that, as c(stem-L) sequences are synthesized, the copy of S10 which is released from the polymerase is transferred to the nascent subunit.

We conclude that during the transcription of the *rrnA* operon the mycobacterial homologues of NusB and S10 form a heterodimer which attaches to the polymerase at Box A_L (nucleotides 38–49, Fig. 2) and remains attached until it is displaced from the polymerase after elongation to nucleotide 1322 and before elongation to nucleotide 1889. A second heterodimer of NusB and S10 attaches at Box A_S (nucleotides 1958–1969) and remains attached at least until elongation to nucleotide 5214. Also, we infer that the NusB and S10 homologues of fast- and slow-growers are very closely related because of the identity of the Box A_L sequence and the near identity of the Box A_S sequence of species of both divisions of mycobacteria.

A homologue of *rrnA* is present in slow-growers

The division into fast-growers and slow-growers is reflected in the phylogeny of mycobacteria inferred from 16S rRNA sequences (see for example Stahl & Urbance, 1990; Rogall, *et al.*, 1990; Stackebrandt & Smida, 1988; Pitulle *et al.*, 1992). In summary, 'The fast-growing mycobacteria (ranging from *M. chelonae* subsp. *abscessus* to *M. thermo-resistibile*) and the slow-growing mycobacteria (ranging from *M. triviale* to *M. paratuberculosis*) are phylogenetically separated. The branching point of the rapidly growing members, close to the root of the mycobacterial subline, indicates that these organisms are phylogenetically older than their slow-growing relatives' (Pitulle *et al.*, 1992).

Fast-growers and slow-growers differ in the number of *rrn* operons per mycobacterium; fast-growers, including *M. phlei* and *M. smegmatis* have two *rrn* operons whereas slow-growers, including *M. tuberculosis* and *M. leprae*, have one operon (Bercovier *et al.*, 1986).

The following tentative hypothesis is based on the assumption that the *rrn* operons of *M. smegmatis* are representative of fast-growers in general, including the most ancient species. In other words, each fast-growing mycobacterium has two *rrn* operons, *rrnA*₁ and *rrnB*₁, specific examples of which are *rrnA* and *rrnB* of *M. smegmatis*. The characteristic features of *rrnA*₁ include a binding site for primer L1 approximately 200 bp upstream from the 5'-end of the 16S rRNA gene and a spacer-1 region of approximately 360 bp. The single *rrn* operon of slow-growers is closely related in primary and secondary

structure to *rrnA_f*, and hence we propose that, at an early stage in the development of the genus, a mycobacterium emerged which had lost a segment of DNA carrying the *rrnB_f* operon and possibly also other genes. The remaining *rrnA_f* operon became the precursor of the *rrnA_s* operon, which is now characteristic of slow-growers, with a leader region of approximately 200 bp and an abbreviated spacer-1 region. Each of the slow-growers we have investigated has an *rrn* operon of the *rrnA_s* family (Ji *et al.*, 1994a, b).

The mechanisms by which *M. smegmatis* and other fast-growing mycobacteria control expression of the two *rrn* operons is not known. Identification of the promoter elements of the two operons and their contribution to the growth of *M. smegmatis* would be helpful. The significance of the differences observed in the promoter regions of *rrnA_f* operons of fast-growers and the *rrnA_s* operon of slow-growers may then become apparent.

Finally, the methods used to amplify the non-coding (leader, spacer-1 and spacer-2) regions of the *rrn* operons of *M. smegmatis* are likely to be generally applicable and so broaden the scope for devising genus-specific and species-specific primers and probes for the identification of mycobacteria.

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