Characterization of the initiator tRNA gene locus and identification of a strong promoter from *Mycobacterium tuberculosis*

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An initiator tRNA gene, *metA*, and a closely linked fragment of a second initiator-tRNA-like sequence, *metB*, from *Mycobacterium tuberculosis* H37Ra have been cloned and characterized. The promoter region of *metA* shows the presence of conserved sequence elements, TAGCCT and TTGGCG, with resemblance to –10 and –35 promoter regions. The deduced sequence of the mature tRNA contains the three unique features of the eubacterial initiator tRNAs represented by (i) a C:U mismatch at position 1:72, (ii) three consecutive base pairs, 29–31G:C39–41 in the anticodon stem, and (iii) a purine:pyrimidine (A:U) base pair at position 11:24 in the dihydrouridine stem. A putative hairpin structure consisting of an 11 bp stem and a three-base loop found in the 3' flanking region is followed by a stretch of T residues and may serve as a transcription terminator. Analysis of the expression of *metA* and of its promoter using chloramphenicol acetyltransferase fusion constructs in *Mycobacterium smegmatis* shows that *metA* is a functional gene driven by a strong promoter. Furthermore, the overexpressed transcripts are fully processed and formylated *in vivo*. The *metB* clone shows the presence of sequences corresponding to those downstream of position 30 of the tRNA. However, the CCA sequence at the 3' end has been mutated to CCG. Interestingly, the 3' flanking sequences of both the genes are rich in GCT repeats. The *metB* locus also harbours a repeat element, IS6110. A method to prepare total RNA from mycobacteria (under acidic conditions) to analyse *in vivo* status of tRNAs is described.

Keywords: mycobacteria, initiator tRNA, promoter, repeats, IS6110

INTRODUCTION

Mycobacteria constitute a group of important micro-organisms responsible for a number of serious public health problems (Kaufmann & van Embden, 1993; McFadden, 1990). While *Mycobacterium tuberculosis* and *Mycobacterium leprae* are the two most harmful pathogens, other species of mycobacteria such as *M. avium*, *M. intracellulare* and *M. kansasii* also cause life-threatening infections in AIDS patients. Chemotherapy to treat mycobacterial infections has given rise to a

multitude of drug-resistant strains. It is therefore important to understand the molecular biology of these organisms so that new therapeutic agents can be designed.

Mycobacteria have G + C-rich genomes up to 70 mol% G + C (Clark-Curtis, 1990). Consequently, their promoter sequences are different from those in *Escherichia coli*, resulting in either poor or no expression of heterologous genes in mycobacteria. Several aspects of protein biosynthesis are also distinct in these organisms, for instance a general bias in the use of G + C-rich codons (Dale & Patki, 1990; Ohama *et al.*, 1987), quantitative differences in tRNA pools of slow- and fast-growing mycobacteria (Bhargawa *et al.*, 1990), and a single rRNA operon in slow-growing and two rRNA operons in fast-growing mycobacteria (Clark-Curtis,

Abbreviations: CAT, chloramphenicol acetyltransferase; oligo, oligodeoxyribonucleotide.

The EMBL accession numbers for the sequences reported in this paper are Y08623 (*metA*) and Y08970 (*metB*).

1990; Ji *et al.*, 1994a, b). Two striking features of mycobacterial tRNAs are the lack of riboT modification at position 54 and the presence of 1-methyladenosine at position 58, making it similar to the eukaryotic tRNA (Vani *et al.*, 1979). More interestingly, a recent study shows that the Ile-tRNA synthetase from *M. tuberculosis* resembles eukaryotic cytoplasmic synthetase rather than eubacterial Ile-tRNA synthetase (Sassanfar *et al.*, 1996). Thus, mycobacteria provide an attractive system to study various aspects of protein biosynthesis.

Here we describe the cloning, sequence analysis and organization of the initiator tRNA gene locus in *M. tuberculosis*. Transcription of the initiator tRNA gene, as analysed in *M. smegmatis*, is driven by a strong promoter and the overexpressed tRNA transcripts are completely processed. In addition, we describe a protocol to isolate total tRNA from *M. tuberculosis* and *M. smegmatis* under acidic conditions for analysing *in vivo* status of the steady-state pools of aminoacylated, formylated and deacylated tRNAs.

METHODS

Bacterial strains and growth media. *M. tuberculosis* H37Ra and *M. smegmatis* SN2 are laboratory strains. *M. smegmatis* mc²155, a high-efficiency transformation strain, was kindly provided by Dr B. Bloom (Albert Einstein College of Medicine, New York). *E. coli* strains TG1 (Amersham) and XL-1 Blue (Stratagene) were used for the recombinant DNA work. *E. coli* was grown in 2YT (Sambrook *et al.*, 1989) and the mycobacteria were grown in modified YK medium (Nagaraja & Gopinathan, 1980). Middlebrook 7H9 broth was used to grow *M. smegmatis* mc²155 to make electro-competent cells, and the transformants were selected on Middlebrook 7H11 agar medium containing 50 µg kanamycin ml⁻¹. Premixed dry media or the individual components were from Difco, Gibco-BRL or Hi-Media (India).

Plasmids. Plasmids pTZ18 and 19R were from Pharmacia. *E. coli*-mycobacteria shuttle vectors pBAK14 and pSD7 series are the constructs from the laboratories of Dr D. Young and Dr A. Tyagi, respectively (Zhang *et al.*, 1991; Dasgupta *et al.*, 1993).

Enzymes, radiochemicals and biochemicals. Enzymes were from Boehringer Mannheim Biochemicals, New England Biolabs or Gibco-BRL. Radiolabelled nucleoside triphosphates and ¹⁴C-labelled chloramphenicol were from Amersham. Other biochemicals of analytical grade were from Sigma, US Biochemicals, Gibco-BRL or Merck.

Oligodeoxyribonucleotides (oligos). These were obtained from the oligo synthesis facility at Centre for Genetic Engineering, Indian Institute of Science, Bangalore, or from Bangalore Genei, Bangalore. An oligo, termed 'anticodon oligo' (5'-CCTCTGGGTTATGAGCCC-3'), complementary to positions 29–46 of the initiator tRNA from *M. smegmatis* SN2 was used to probe Northern blots, colony lifts and the Southern blots.

Preparation of total tRNA under acidic conditions. Total tRNA was prepared as described previously (Varshney *et al.*, 1991b). However, the rigid and lipid-rich cell wall of mycobacteria necessitated significant modifications to the protocol, described as follows.

For *M. smegmatis*, YK broth (100 ml) was inoculated with 1 %

(v/v) inoculum and grown for 20 h at 37 °C with vigorous shaking (growth media for the various transformants were supplemented with 50 µg kanamycin ml⁻¹). The culture was chilled on ice and the rest of the procedures carried out at 4 °C. The bacteria were harvested by centrifugation, suspended in 4 ml SE (0.3 M sodium acetate pH 4.5, 10 mM Na₂EDTA), and mixed with an equal volume of SE-saturated phenol by vortexing for 1 min. The suspension was ultrasonicated for 1 min using a sonicator (LSL Secfroid model W380) fitted with a microprobe (output setting 4, 2 s pulses, 50 % duty cycle), vortexed again for 1 min and centrifuged. The aqueous phase was collected and re-extracted with SE-saturated phenol by vortexing for 1 min and centrifuged. Total tRNA from the aqueous layer was purified by two successive steps of ethanol precipitation and dissolved in 0.25 ml 10 mM sodium acetate (pH 4.5), 1 mM Na₂EDTA. RNA was estimated by measuring A₂₆₀ and the samples were stored at -70 °C.

For *M. tuberculosis*, between five and ten sections (approx. 2 mm diameter) of *M. tuberculosis* surface culture were used to seed 100 ml YK medium (without Tween 80) in a 500 ml conical flask and grown for 10–12 d at 37 °C as stationary surface culture. Subsequently, the culture was supplemented with 0.2 % Tween 80 (v/v) and grown for 24 h under shaking conditions. The cells were harvested and processed as above.

***In vitro* aminoacylation/formylation.** The total tRNA (A₂₆₀ 0.75) was deacylated by incubating in 0.1 M Tris/HCl (pH 9.5) for 30 min at 37 °C and ethanol precipitated. Deacylated total tRNA was subjected to aminoacylation and formylation (Varshney *et al.*, 1991b).

Electrophoresis of total tRNA on acid urea gels and Northern blotting. Total tRNA (A₂₆₀ 0.25) was separated on acid urea gels, electro-blotted to a nylon membrane (Hybond N⁺, Amersham) and probed with the anticodon oligo (Varshney *et al.*, 1991b).

Preparation of genomic DNA. *M. smegmatis* SN2 or *M. tuberculosis* H37Ra cells from 1 l cultures were harvested, frozen at -70 °C, thawed and suspended in 30 ml GTET (20 mM Tris/HCl, pH 8.0; 50 mM glucose; 10 mM EDTA; 5 %, v/v, Triton X-100). To this, 60 mg lysozyme was added and the mixture left for 10 h at 37 °C. It was then treated with 6 mg proteinase K in the presence of 1 % SDS (w/v) at 65 °C for 5 h. The rest of the procedure was as described previously (Varshney *et al.*, 1988).

Southern blotting. Genomic DNA was digested with the restriction endonucleases, fractionated on agarose gels using Tris/borate/EDTA buffer (Sambrook *et al.*, 1989), transferred to nylon membranes (Nytran, Schleicher & Schuell) by vacuum blotting using 0.4 M NaOH (Reed & Mann, 1985) and hybridized to 5' ³²P-end-labelled DNA probes (Chaconas & van de Sande, 1980).

Hybridization and autoradiography. Hybridization of the nucleic acids fixed to the nylon membranes was as described previously (Varshney *et al.*, 1991b) except that the SET buffer was replaced with SSC buffer (Sambrook *et al.*, 1989). Hybridizations using DNA oligos as probes were done at 42 °C for 18–20 h and the filters were washed with decreasing concentrations of SSC (4 ×, 3 × and 2 ×, 30 min each) at 42 °C in the presence of 0.2 % (w/v) SDS. Filters were exposed to Konica X-ray films (Computer Graphics, India) using hyperscreens (Amersham) at -70 °C.

Recombinant DNA techniques. Standard techniques (Sambrook *et al.*, 1989) were followed. Electroporation of *M. smegmatis* mc²155 with pBAK14- or pSD7-based vectors and selection of the transformants were as described by Zhang *et al.* (1991).

Preparation and screening of partial genomic libraries. For cloning of the 0.34 kb *Ava*I fragment, genomic DNA (5 µg) was digested with *Ava*I (6 units) and separated on low melting agarose (SeaPlaque, FMC). DNA fragments corresponding to 0.3–0.4 kb were cloned into the *Ava*I site of pTZ19R. Transformants (approx. 400 colonies) were screened by colony hybridization using 5' ³²P-end-labelled anticodon oligo.

For cloning of the 1.7 kb *Bam*HI fragment, genomic DNA (150 µg) was digested with excess *Bam*HI (750 units) and fractionated on a 10–30% (w/v) sucrose density gradient (Sambrook *et al.*, 1989). Fractions enriched in 1.7 kb DNA fragments were cloned into the *Bam*HI site of pTZ19R. Transformants (approx. 900 colonies) were screened by colony hybridization as above.

DNA sequence analysis. Single-stranded templates obtained from pTZ18R or 19R constructs were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 (US Biochemicals).

Construction of the initiator tRNA promoter–chloramphenicol acetyltransferase (CAT) gene fusion. The promoter region of the *metA* gene was PCR amplified using the reverse sequencing primer (5'-CAGGAAACAGCTATGAC-3') corresponding to the vector sequence upstream of the multiple cloning site and a primer (5'-CGAGCGGATCCAACCG-CGTC-3') complementary to nucleotides 86–106 of the tRNA gene (see Fig. 3). Positions 6 and 8 of the second primer carried mutations to generate a *Bam*HI site, and position 13, which corresponded to the tRNA sequence, was a C-to-A change inadvertently introduced during synthesis (the positions are shown in bold italics). The PCR reaction using *metA* template and 10 pmol of each of the primers was performed (Saiki *et al.*, 1988) using *Taq* DNA polymerase (Gibco-BRL). Each of the 25 cycles was incubated at 94 °C for 1 min, 40 °C for 30 s and 68 °C for 30 s, and the reaction was completed with incubation at 68 °C for 5 min. The PCR product was ethanol precipitated, digested with *Bam*HI to release a 0.1 kb fragment (upstream *Bam*HI site from the multiple cloning sites of the vector and the downstream *Bam*HI site from the second primer) and cloned into the *Bam*HI site immediately upstream of the promoterless CAT gene in pSD7 (Dasgupta *et al.*, 1993). Recombinants in both orientations of the monomeric insert were selected by restriction mapping, verified by DNA sequencing with a primer (5'-CGGTCTGGTTATAGT-ACA-3') complementary to the CAT gene open reading frame and transformed into *M. smegmatis* mc²155 (Zhang *et al.*, 1991).

Preparation of cell-free extracts and CAT assays. Cells from 18 h cultures of the various transformants were harvested, washed with TME (20 mM Tris/HCl, pH 8.0; 1 mM β-mercaptoethanol; 1 mM Na₂EDTA), suspended in 0.5 ml of the same buffer and disrupted using the sonicator fitted with a microprobe for 1 min (50% duty cycle, 5 s pulses). Cellular debris was removed by centrifugation and CAT activities in the extracts (0.5 and 1.0 µg total protein) were assayed in the presence of excess substrates (Varshney *et al.*, 1991a), expressed as nmol product formed min⁻¹ (mg total protein)⁻¹.

RESULTS

Oligodeoxyribonucleotide probe (anticodon oligo) and its use in Northern and Southern blot analyses

The nucleotide sequence of the initiator tRNA of *M. smegmatis* SN2 as determined by the RNA fingerprinting technique is known (Vani *et al.*, 1984; Fig. 1). We designed an anticodon oligo complementary to positions 29–46. To ascertain the specificity of the oligomeric probe, we exploited the fact that the prokaryotic initiators can exist in three distinct forms (deacylated, aminoacylated and formylated). Total tRNA from *M. smegmatis* and *M. tuberculosis* was subjected to *in vitro* aminoacylation/formylation and fractionated on acid urea gels (Varshney *et al.*, 1991b). On these gels, the aminoacylated tRNA migrates more slowly than the deacylated tRNA and the formylated tRNA migrates at an intermediate rate. Northern blot analysis using anticodon oligo detected a single band in each of the lanes 1–3 and 5–7 (Fig. 2a). Expected relative mobilities of the bands unequivocally confirmed the specificity of the oligomeric DNA probe in that it detected only the initiator tRNA. In addition, the results showed that *in vivo*, initiator tRNA in *M. smegmatis* and *M. tuberculosis* exists in the formylated form (Fig. 2a, lanes 4 and 8).

As our aim was to clone and characterize *M. tuberculosis* initiator tRNA gene(s), we used the anticodon oligo to probe the Southern blots of genomic DNA digested with various restriction endonucleases (Fig. 2b). Two distinct bands, of 6.0 kb and 1.7 kb (lane 1, *Bam*HI), or 1.6 kb and 0.34 kb (lane 3, *Ava*I), were seen. Since the probe was only 18 nucleotides in size, devoid of either the

Fig. 1. Clover leaf structure of *M. smegmatis* tRNA^{fMet}. Boxed regions represent the highly conserved features of the prokaryotic initiator tRNAs. The anticodon oligo, 5'-CCTCTGGGTTATGAGCCC-3', is complementary to nucleotide positions 29–46.

Fig. 2. (a) Northern blot analysis of the total tRNA prepared from *M. tuberculosis* H37Ra (MT) and *M. smegmatis* SN2 (MS). Total tRNA preparations (A_{260} approx. 0.25) were fractionated on an acid urea gel, transferred to nylon membrane and hybridized to the radiolabelled anticodon oligo (see legend to Fig. 1). Lanes 1 and 5, deacylated tRNA; lanes 2 and 6, *in vitro* formylated tRNA; lanes 3 and 7, *in vitro* aminoacylated tRNA; lanes 4 and 8, total tRNA from the two mycobacteria prepared under acidic conditions. (b) Southern blot analysis of the genomic DNA of *M. tuberculosis* H37Ra. Approximately 5 μ g DNA was digested with the restriction endonucleases as indicated, separated on 0.9% agarose gel, transferred to nylon membrane and hybridized to the 5' 32 P-end-labelled anticodon oligo (see legend to Fig. 1). The sizes of the various fragments are indicated.

*Bam*HI or the *Ava*I sites, these results suggested that *M. tuberculosis* contains at least two initiator tRNA loci. The presence of a single band of approximately 8.5 kb in *Eco*RI digest indicated that the two loci are linked. Interestingly, in the *Bam*HI digest, the 1.7 kb band was less strong compared to the 6.0 kb band (lane 1). The weak intensity of the signal could not be due to inefficient retention of the low molecular mass DNA fragments on the nylon membrane as in the *Ava*I digest, a band of the lower size (0.34 kb) was more intense than the 1.6 kb band (lane 3).

Cloning and sequence analysis of *metA*

Screening of a partial genomic library of *Ava*I fragments yielded two positive clones with identically sized inserts. One of the clones was further analysed by determining its DNA sequence, shown in Fig. 3. The sequence revealed a region of 77 nucleotides (tDNA) from position 88 to 164 which matched completely the sequence of *M. smegmatis* initiator tRNA (Vani *et al.*, 1984). Upstream of the tDNA sequence, two hexameric sequences, TAGCCT and TTGGCG, separated by 18 nucleotides (positions 46–51 and 70–75, respectively, shown in bold in Fig. 3), are present. A potentially strong hairpin structure with a stem 11 bp long and a three-base loop is located between positions 174 and

198. This structure is followed by a run of Ts and could serve as a transcription terminator.

Cloning and sequence analysis of *metB*

Screening of a partial genomic library enriched for approximately 1.7 kb *Bam*HI fragments with the anticodon oligo yielded two positive clones containing identically sized inserts. One of these was completely sequenced and found to be 1666 bp in size. To our surprise, the insert showed only a short stretch of sequence (from 638 to 682) with complementarity to positions 31–75 of the initiator tRNA (standard tRNA numbering, Rich & RajBhandary, 1975; Sprinzl *et al.*, 1989). As the anticodon oligo is complementary to tRNA sequence between positions 46 and 29, the insert lacks two of the nucleotides complementary to the 3' end of the probe and could be responsible for differential band intensities seen in Fig. 2(b) (lanes 1 or 3).

The *metB* locus harbours a repeat element

Southern blot analysis of the genomic DNA from *M. tuberculosis* H37Ra using the *metB* clone revealed several strongly hybridizing bands (data not shown) suggesting the presence of a repeat element in the *metB* clone. Comparison of the *metB* locus sequence to the

Fig. 5. Restriction map of *E. coli*-mycobacteria shuttle vector pSD7 containing a promoterless CAT reporter gene.

does not cross-hybridize to the mycobacterial tRNA probe) on the same vector (lane 3), the host with a plasmid-borne *metA* gene showed a markedly increased hybridization signal (lane 2). This observation shows that *metA* contains an active tRNA^{fMet} gene. To ascertain the *in vivo* status of the overexpressed tRNA, Northern blot analysis using acid urea gel was performed (Fig. 4c). Total tRNA prepared under acidic conditions from a vectorless host served as a marker for fMet-tRNA^{fMet} (Fig. 4c, lane 1; also see Fig. 2a). The overexpressed tRNA comigrated with the fMet-tRNA^{fMet} marker (Fig. 4c, lanes 3 and 1), suggesting its complete formylation. Treatment under alkaline conditions (Fig. 4c, lane 2), as expected, resulted in its slightly faster mobility due to deacylation, confirming the formylated status of the tRNA in lane 3. The presence of single bands in lanes 2 and 3 (Fig. 4c) also established that the overexpressed tRNA was completely processed to the mature form.

Fusion of the *metA* promoter to the CAT gene and analysis of the promoter strength

The promoter sequence of the *metA* gene was PCR amplified and cloned in both orientations into pSD7 immediately upstream of the promoterless CAT reporter gene (Fig. 5) and transformed into *M. smegmatis* mc²155. We also used a previously characterized strong mycobacterial promoter, S16 from *M. smegmatis*, cloned into the same vector and in the same position (Dasgupta *et al.*, 1993), as a control to obtain the relative strength of the *metA* promoter. Cell-free extracts from the various transformants were prepared and assayed for CAT activity. The activity assays show that *metA* contains a promoter which is stronger than the S16 promoter: 1693 nmol product min⁻¹ (mg total protein)⁻¹ for *metA*-2 versus 1145 nmol product min⁻¹ (mg total protein)⁻¹ for pSD7-S16. No CAT activity above the background [pSD7: 17 nmol min⁻¹ (mg total protein)⁻¹] could be detected when the *metA* promoter was cloned

in the wrong orientation [*metA*-1: 19 nmol min⁻¹ (mg total protein)⁻¹], suggesting the specificity and the directionality of the promoter.

DISCUSSION

We describe cloning and characterization of an initiator tRNA gene, *metA*, and a closely linked locus, *metB*, from *M. tuberculosis* H37Ra. The *metA*-encoded tRNA shows the presence of all the three highly conserved features of the eubacterial initiators (Sprinzl *et al.*, 1989) which include a mismatch at the top of the acceptor stem (1:72), a purine:pyrimidine base pair at position 11:24 and the three consecutive GC base pairs in the anticodon stem (positions 29–31:39–41) (Fig. 1). We also show that the sequence of the initiator tRNA from *M. tuberculosis* H37Ra is identical to that of *M. smegmatis* SN2 (Figs 1 and 3). Although our present studies do not directly rule out the possibilities of any differences at the level of base modifications, comigration of the various forms of the initiator tRNA from the two organisms (Fig. 2a) could indicate that they contain similar base modifications (Mangroo *et al.*, 1995). The Northern blot analysis of the total tRNA prepared under acidic conditions from *M. tuberculosis* and *M. smegmatis* shows that *in vivo* the tRNA exists in the formylated form (Figs 2a and 4c). Hence, even though mycobacterial initiator tRNAs contain some features of the eukaryotic initiators, they still use formylated tRNA in initiation. A previous report also showed the presence of the formylating enzyme in mycobacteria (Deobagkar & Gopinathan, 1978).

Based on the comparison of –35 and –10 regions of the mycobacterial genes, we have identified similar sequences in the *metA* gene. In *metA* these sequences, represented by TTGGCG and TAGCCT respectively, are composed of the most commonly occurring sequences in the other mycobacterial genes (Ji *et al.*, 1994a, b; Kempell *et al.*, 1992; Baird *et al.*, 1989; Mahethiralingam *et al.*, 1993; Madhusudan & Nagaraja, 1995; Kenney & Churchward, 1996; Nesbit *et al.*, 1995). A recent study, where a large number of randomly cloned mycobacterial promoters were analysed, suggested that the mycobacterial promoters do not possess a consensus sequence in the –35 region (Bhaskar *et al.*, 1996). Hence, it will be important to analyse the sequence in the –35 region in the *metA* promoter. The assigned –10 and –35 regions in the *metA* gene are separated by 18 bp. A distance of 17 ± 1 bp has been found to be optimal in *E. coli* promoters (Hawley & McClure, 1983; Reznikoff *et al.*, 1985). A fusion gene construct between the *metA* promoter and a promoterless CAT gene showed that transcription of *metA* is driven by a strong promoter. In *E. coli*, there are a total of four functional genes (Ishii *et al.*, 1984; Kenri *et al.*, 1994) to ensure sufficient pools of the initiator tRNA. Hence, the presence of a strong promoter in front of the single functional initiator tRNA gene in *M. tuberculosis* could be of significance. Finally, the cloning and characterization of an active initiator tRNA gene (*metA*)

should now enable us to analyse the structure and function of the mycobacterial initiator tRNA.

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