Organization and copy number of initiator tRNA genes in slow- and fast-growing mycobacteria

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We have previously reported the isolation and characterization of a functional initiator tRNA gene, *metA*, and a second initiator tRNA-like sequence, *metB*, from *Mycobacterium tuberculosis*. Here we describe the fine mapping of the initiator tRNA gene locus of the avirulent (H37Ra) and virulent (H37Rv) strains of *M. tuberculosis*. The genomic blot analyses show that the 1·7 kb (harbouring *metB*) and the 6·0 kb *Bam*HI (harbouring *metA*) fragments are linked. Further, sequencing of a portion of the 6·0 kb fragment, in conjunction with the sequence of the 1·7 kb fragment confirmed the presence of an IS*6110* element in the vicinity of *metB*. The IS element is flanked by inverted (28 bp, with 3 contiguous mismatches in the middle) and direct (3 bp) repeats considered to be the hallmarks of IS*6110* integration sites. The organization of the initiator tRNA gene locus is identical in both the H37Ra and H37Rv strains and they carry a single copy of the functional initiator tRNA gene. Interestingly, the fast growing *Mycobacterium smegmatis* also bears a single initiator tRNA gene. This finding is significant in view of the qualitative differences in total tRNA pools and the copy number of rRNA genes in the fast- and slow-growing mycobacteria. Finally, we discuss hypotheses related to the origin of *metB* in *M. tuberculosis*.

1. Introduction

Mycobacteria consist of a closely related group of slow and fast growing microorganisms. The slow growing pathogenic *Mycobacterium tuberculosis* and *M. leprae*, and the opportunistic pathogens like *M. avium*, *M. intracellulare* and *M. kansasi* afflict immunocompromised patients. The fast growers include nonpathogens like *M. smegmatis*, and pathogens such as *M. chelonae* and *M. fortuitum*. The growth rate of an organism is generally correlated to the rate of protein synthesis which in turn is dependent upon the abundance of ribosomes and the other cellular components associated with protein biosynthesis (Bremer and Dennis 1987). The premise is supported by the presence of two ribosomal RNA operons in the fast growing mycobacteria as opposed to one in the slow growers (Bercovier *et al* 1986; Ji *et al* 1994a,b; Musser 1995). Also, qualitative differences exist in the total tRNA pools between *M. tuberculosis* and *M. smegmatis* (Bhargava *et al* 1990). Such unusual features of the protein synthesis machinery in the slow- and fast-growing mycobacteria offer attractive models to study the mechanistic aspects of protein biosynthesis. Previously, we reported that *M. tuberculosis* has a single functional initiator tRNA gene (Vasanthakrishna *et al* 1997). To our knowledge, characterization of initiator tRNA genes from no other mycobacterial species has been reported so far.

Initiation is a major rate limiting step in protein biosynthesis. It is therefore not surprising that *Escherichia coli* has evolved with four functional initiator tRNA genes (Ishii *et al* 1984; Kenri *et al* 1994). We recently described the isolation and characterization of a 0·34 kb *Ava*I fragment containing a functional initiator tRNA gene, *metA*, and a 1·7 kb *Bam*HI fragment containing an initiator tRNA-like sequence, *metB* from *M. tuberculosis* H37Ra (Vasanthakrishna *et al* 1997). Here we complete the description of the initiator tRNA locus in *M. tuberculosis* H37Ra and H37Rv by showing that *metA* and *metB* are located on contiguous *Bam*HI fragments, sepa-

Keywords. *metA*; *metB*; IS*6110*; integrative elements

rated by an insertion element, IS*6110*. The element is flanked by inverted and direct repeats characteristic of IS*6110* integration sites (Thierry *et al* 1990). In addition, we speculate on the origin of *metA* and *metB* tDNA sequences. Surprisingly, *M. smegmatis* used as a representative of fast growers shows the presence of a single initiator tRNA gene. More importantly, the isolation of *metA* in a larger genomic fragment would be useful to pursue the mutational analysis of the initiator tRNA gene by allelic exchange with the chromosomal copy (Pelicic *et al* 1997).

2. Materials and methods

2. *Bacterial strains and growth media*

M. tuberculosis H37Ra and *M. smegmatis* SN2 are laboratory strains and were grown in modified Youmans and Karlson's (YK) medium (Nagaraja and Gopinathan 1980). *E. coli* strains TG1 (Amersham) and XL1-blue (Stratagene) were grown in 2YT medium (Sambrook *et al* 1989). Media components were procured from Hi-Media, Mumbai.

2.2 *Plasmids, enzymes, radioisotopes and biochemicals*

Plasmids pTZ-18R and -19R were from Pharmacia Amersham Biotech. Restriction endonucleases were from New England Biolabs or Gibco-BRL. Radiolabelled nucleoside triphosphates were purchased from Pharmacia Amersham Biotech. Other biochemicals of analytical grade were from Sigma, US Biochemicals, Gibco-BRL or Merck.

2.3 *Oligodeoxyribonucleotides (oligos)*

The oligos were obtained from Bangalore Genei Pvt. Ltd., Bangalore, purified on 15% acrylamide (w/v) 8 M urea gels (Maxam and Gilbert 1980) and desalted by gel filtration on Sephadex G-50 (Pharmacia Amersham Biotech.). An oligo, termed 'anticodon oligo', 5'-CCTCTGGGTTATGAGCCC-3' complementary to positions 29–46 of the mycobacterial initiator tRNA (figure 6A) was used in colony hybridization and genomic Southern analyses. Oligonucleotide, 5'-CGAGCGGATC-CAACCCGCGTC-3' corresponding to positions -2 to 19 (Vasanthakrishna *et al* 1997, figure 6A) was used for probing a recombinant plasmid blot.

2.4 *Preparation of genomic DNA*

Genomic DNA from *M. smegmatis* SN2 and *M. tuberculosis* H37Ra were prepared as described (Vasanthakrishna *et al* 1997) and the genomic DNA of *M. tuberculosis* H37Rv was a kind gift from Dr V Nagaraja.

2.5 *Southern blotting*

Genomic DNA was digested with the restriction endonucleases, separated on agarose gels using Tris-borate-EDTA buffer (Sambrook *et al* 1989), transferred to nylon membranes (Nytran, Schleicher and Schuell) by vacuum blotting using 0·4 M NaOH (Reed and Mann 1985).

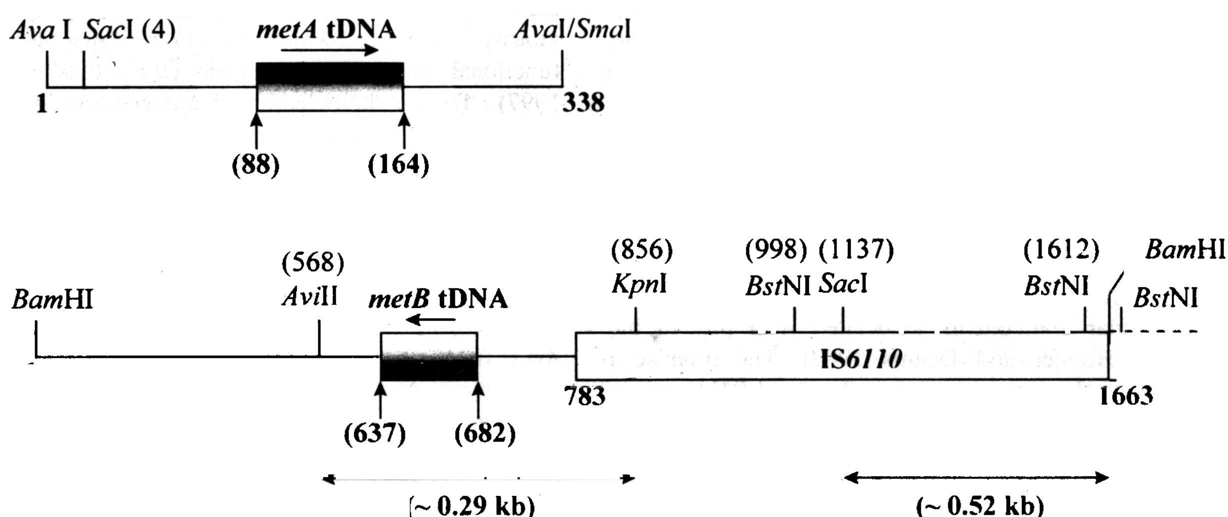

Figure 1. Restriction maps of the 0·34 kb *Ava*I fragment containing *metA* gene (A) and, the 1·7 kb *Bam*HI DNA fragment containing *metB* and part of IS*6110* sequence (B). The vertical arrows below the shaded boxes indicate the nucleotide positions of tDNA sequences. Horizontal arrows above the boxes indicate the orientation of *metA* and *metB* genes. Nucleotide position 783 indicates the beginning of IS*6110*. The 0·29 and 0·52 kb probes used for various Southern blot analyses are as shown. The broken lines indicate the sequence of IS*6110* missing from the 1·7 kb clone.

2.6 *Preparation of radiolabelled of DNA probes*

Restriction fragments were labelled by random priming (Sambrook *et al* 1989) in the presence of [α-^{32}P]dCTP (3000 to 6000 Ci mmol^{-1}, Amersham) using hexanucleotide primers (New England Biolabs). The DNA oligomers used as probes for Southern and colony hybridizations were 5′-end labelled using [γ-^{32}P]ATP (3000-6000 Ci mmol^{-1}, Amersham) and T4-polynucleotide kinase (Chaconas and van de Sande 1980).

2.7 *Hybridization and autoradiography*

Hybridization of nucleic acids fixed to the nylon membranes was performed as described (Vasanthakrishna *et al* 1997). Hybridizations using DNA oligomer probes were done at 40°C for 14 to 16 h and the filters were washed with SSC in the following order – 4 ×, 3 ×, 2 × for 30 min each; at 37°C in the presence of 0·1% (w/v) SDS and exposed to Konica X-ray films (Computer Graphics Ltd., India) at –70°C.

2.8 *Recombinant DNA techniques*

Standard techniques (Sambrook *et al* 1989) were followed.

2.9 *Preparation and screening of partial genomic library*

Genomic DNA (20 μg) was digested with *Bam*HI and separated on an agarose gel. DNA fragments correspond-

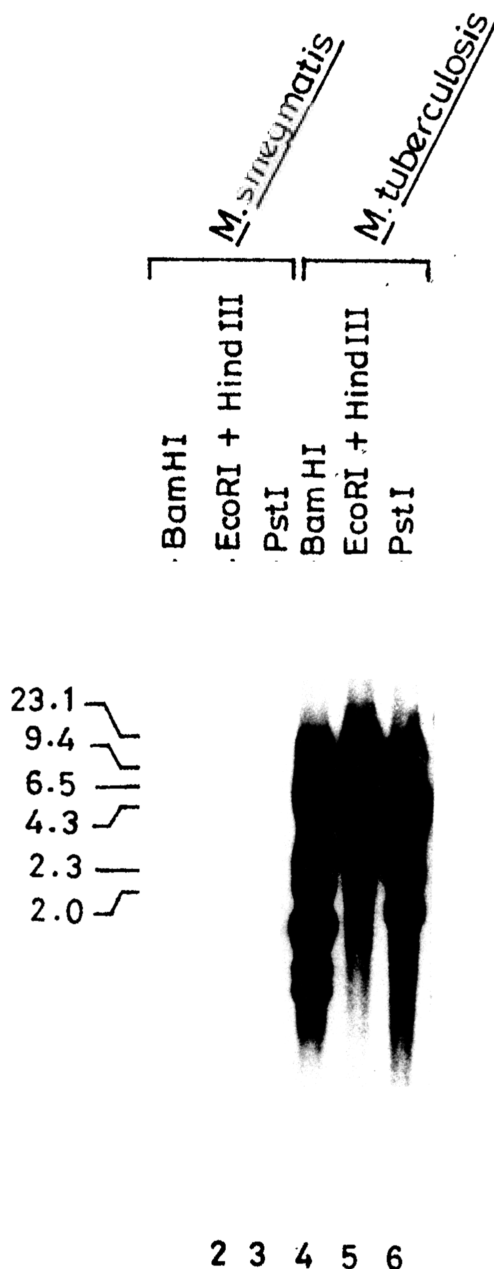

Figure 2. Genomic blot analysis of *M. smegmatis* SN2 (lanes 1–3) and *M. tuberculosis* H37Ra DNA (lanes 4–6) using the 0·29 kb *Avi*II to *Kpn*I fragment as probe (refer figure 1B). Restriction endonucleases used are shown on the top of the lanes. To enable detection of the initiator tRNA gene(s) in *M. smegmatis*, post hybridization washings were performed at 60°C. Remaining conditions were as detailed in § 2. *Hind*III digested λ-DNA was used as marker.

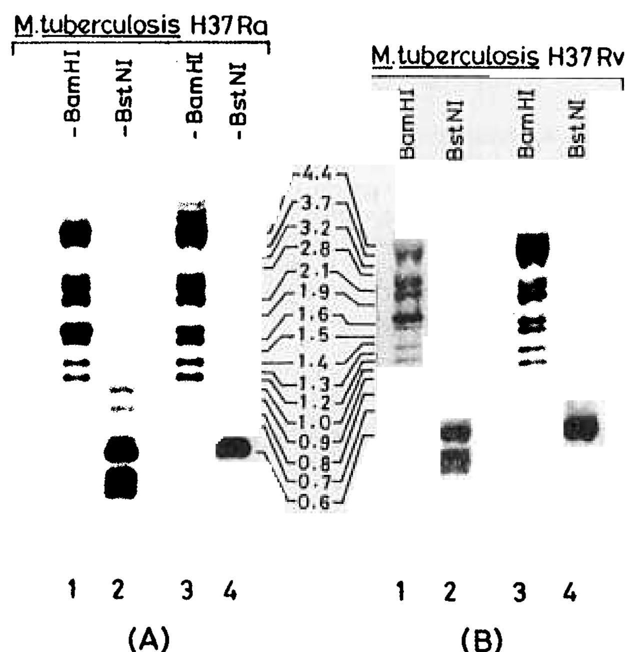

Figure 3. The presence of repeat elements in *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv: Southern blot analysis of *Bam*HI and *Bst*NI digested genomic DNA from *M. tuberculosis* H37Ra (**A**) or *M. tuberculosis* H37Rv (**B**) using the 0·29 kb *Avi*II to *Kpn*I probe (lanes 1 and 2); and the 0·52 kb *Sac*I to *Bam*HI probe (lanes 3 and 4). The probes used are indicated in figure 1(B). *Hind*II and *Hind*III digested λ-DNA was used as marker.

ing to 5·5 to 7·0 kb were spin eluted (5000 g for 10 min in a microfuge) by placing the corresponding gel piece over polyester wool in a 0·5 ml Eppendorf tube with a punctured bottom, fitted into 1·5 ml Eppendorf tube. DNA was extracted with phenol/chloroform (1 : 1 v/v), precipitated with ethanol and cloned into *Bam*HI site of pTZ18R. Recombinants were screened by colony hybridization using [^{32}P]-labelled 'anticodon oligo'.

2.10 *DNA sequence analysis*

DNA sequencing was performed by dideoxy chain termination method (Sanger *et al* 1977) using either Sequenase version 2·0 (US Biochemicals) or the DNA Cycle Sequencing System (Gibco BRL).

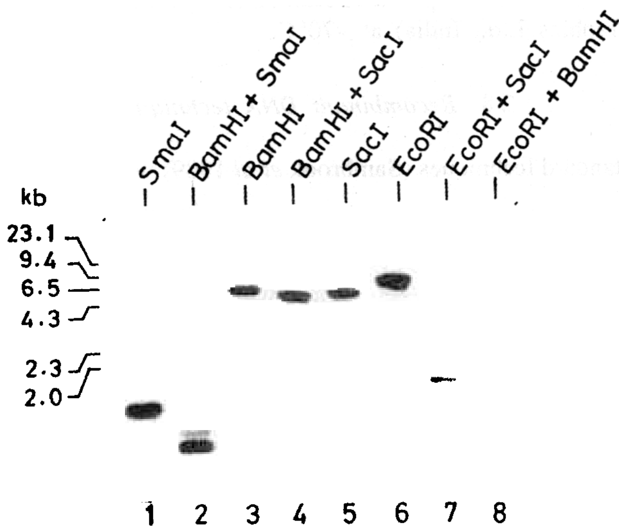

Figure 4. Southern blot of *M. tuberculosis* H37Ra genomic DNA digested with various restriction endonucleases and probed with the 0·34 kb *Ava*I fragment (harbouring *met*A, figure 1A). Approximate sizes of the various fragments (kb) are as follows. Lane 1, 3·2 and **1·4**; lane 2, 1·0 and **0·8**; lane 3, **6** and 1·7; lane 4, **5·5** and 1·1; lane 5, **6** and 1·8; lane 6, 8·5; lane 7, **6** and 1·8; and lane 8, **6** and 1·7. (The sizes of the *metA* containing fragments are indicated in bold).

3. Results and discussion

3 *metA and metB loci*

Southern blot analysis of *Bam*HI digested genomic DNA of *M. tuberculosis* H37Ra using the 'anticodon oligo' showed two hybridizing bands of approximately 6·0 kb and 1·7 kb harbouring *metA* and *metB*, respectively (Vasanthakrishna *et al* 1997). Further analyses showed that the 0·34 kb *Ava*I fragment represented the same initiator tRNA gene (*metA*) as the one present in the 6·0 kb *Bam*HI fragment. Isolation and sequencing of the 0·34 kb *Ava*I (exact size, 0·338 kb) and the 1·7 kb *Bam*HI (exact size, 1.663 kb) genomic fragments harbouring *metA* and *metB* respectively has been described (EMBL accession numbers Y08623 and Y08970; Vasanthakrishna *et al* 1997). Figure 1 shows the restriction maps of the two clones. The sequence of *metA* revealed a region of 77 nucleotides (tDNA) from position 88 to 164 which is identical with that of *M. smegmatis* initiator tRNA sequence determined by RNA finger printing (Vani *et al* 1984). Surprisingly, the 1·7 kb *Bam*HI fragment showed only a short region of sequence complementary to positions 31–75 of the initiator tRNA (standard tRNA numbering, Rich and RajBhandary 1975; Sprinzl *et al* 1989). Sequence homology search of the *metB* locus in the EMBL nucleotide data bank showed absolute identity (> 99·6%) in the sequence downstream of position 782 in the *metB* clone to an insertion element, IS*6110* or IS*987*. The IS*6110* is a repeat element of 1361 bp with a copy number of up to 20 in the *M. tuberculosis* complex (Poulet and Cole 1995 and references cited therein; Thierry *et al* 1990). The *Bam*HI site at the end

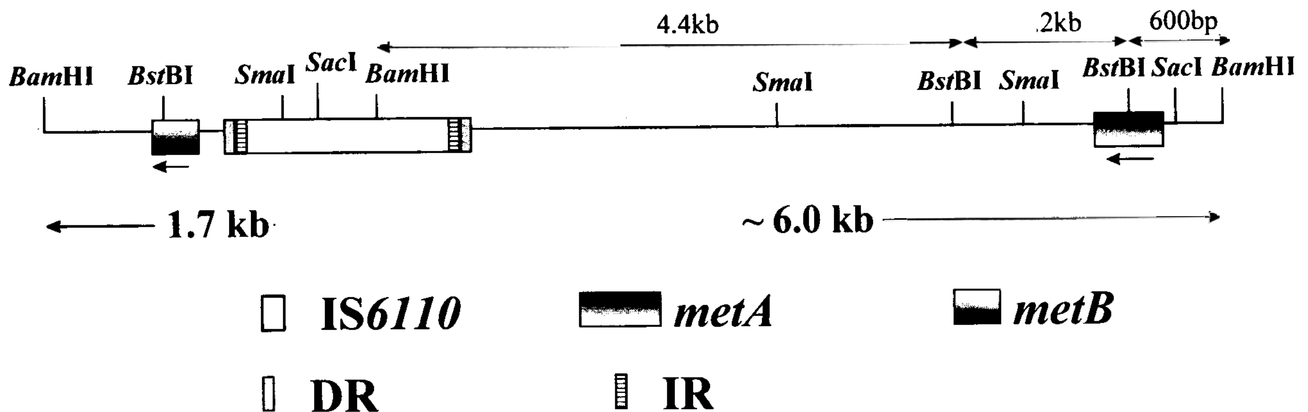

Figure 5. Linkage map of 6·0 kb and 1·7 kb *Bam*HI fragments. Relevant restriction sites are as indicated. The shaded boxes refer to the tDNA sequences of *metA* and *metB*. IS*6110* is represented by an open box flanked by the inverted (IR) and direct (DR) repeats. Sizes of the fragments released by *Bst*BI and *Bam*HI digestion of the 6·0 kb fragment (figure 6C) are as shown.

of the cloned 1·7 kb fragment (position 1663) corresponds to the *Bam*HI site of the IS element at position 881 (IS*6110* numbering), hence the *metB* clone lacks the IS*6110* sequence downstream of the *Bam*HI site.

3.2 *Organization of IS6110, metA and metB*

Southern blot analysis of the genomic DNA from *M. smegmatis* SN2 and *M. tuberculosis* H37Ra (figure 2) using a 0·29 kb *Avi*II to *Kpn*I fragment from the 1·7 kb clone (figure 1B) showed several strong bands in *M. tuberculosis* (figure 2, lanes 4–6), suggesting multiple copies of IS*6110* in the genome. Since *M. smegmatis* does not harbour IS*6110* (Poulet and Cole 1995) the distinct bands in lanes 1–3 most likely correspond to the initiator tRNA gene(s).

To further investigate the copy number of the IS element, we used a second probe (0·52 kb) from a region internal to IS*6110* (*Sac*I-*Bam*HI fragment, see figure 1B). Southern blot analyses of *Bam*HI and *Bst*NI digested genomic DNA from *M. tuberculosis* H37Ra and H37Rv using the 0·29 kb probe are shown in lanes 1 and 2 (figure 3A,B) and those to 0·52 kb probe in lanes 3 and 4 (figure 3A,B). Comparison of lanes 1–3 in figure 3(A) with the corresponding lanes in figure 3(B) indicates a similar pattern of hybridization in the *Bam*HI and *Bst*NI genomic digests of *M. tuberculosis* H37Ra and H37Rv. Since these sites are also present within IS*6110* (figure 1B), the results suggest identical organization of the repeat element in the two strains. Further, as IS*6110* contains three internal *Bst*NI sites, digestion of the genomic DNA with *Bst*NI is expected to release internal fragments of approximately 0·61 kb and 0·05 kb from all copies of IS*6110*. As expected, only the 0·61 kb fragment was detected by hybridization to 0·52 kb probe (lane 4, figure 3A,B) which further establishes that all the bands in the *Bam*HI digests (lanes 1 and 3) belong to IS*6110*. Also, strong hybridization of 0·29 kb probe (which contains *metB*) to the 1·7 kb band in the *Bam*HI digests [compare figure 3(A) (lane 1) to figure 3(B) (lane 1)] suggests identical organization of *metB* in both the strains of *M. tuberculosis*.

Southern blot of *M. tuberculosis* H37Ra DNA probed with the 0·34 kb *Ava*I fragment (figure 1A) to distinguish *metA* (strong signal) from *metB* (weak signal) has allowed further analysis of the linkage of *metA* and *metB* (figure 4). A single band of ~ 8·5 kb in the *Eco*RI digest (lane 6) shows that *metA* and *metB* loci are linked over a distance of 8·5 kb. The sizes of the two bands of 1·7 and 6·0 kb in the *Bam*HI digest (lane 3) remain unaltered upon further digestion with *Eco*RI (lane 8). This observation together with the combined size of these fragments (7·7 kb) suggests that they are located within the 8·5 kb *Eco*RI fragment. To a first approximation, the 1·7 kb and the 6·0 kb *Bam*HI DNA fragments should be adjacent to each other. Digestion with *Sma*I released *metA* in an ~ 1·4 kb fragment (lane 1), which upon further digestion with *Bam*HI generated a band of ~ 0·8 kb (lane 2). As a *Sma*I site is found immediately downstream of *metA* (figure 1A), these results suggest that *metA* is located within 0·8 kb from one end of the 6·0 kb *Bam*HI fragment. Sizes of the various fragments harbouring *metB* wherever deducible are as expected (lanes 2, 3, 4 and 8). Fragments of ~ 1·8 kb encompassing *metB* (lanes 5 and 7) are most likely due to the presence of a *Sac*I site ~ 0·7 kb upstream of position 1 of the 1·7 kb *Bam*HI fragment.

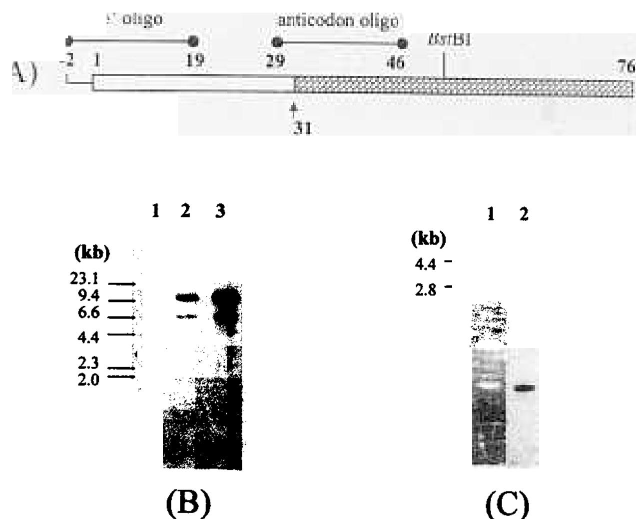

Figure 6. Southern blot analyses. (**A**) The box (1–76) indicates the *metA* tRNA while the cross-hatched region (31–76) shows the overlap with *metB* tRNA. The positions of the 5′ end DNA oligomer (positions -2 to 19) and the anticodon oligo (positions 29–46) used as probes for Southern analysis are indicated on the top. (**B**) Southern blot of the genomic DNA from *M. smegmatis* (lane 1) *M. tuberculosis* H37Ra (lane 2) and H37Rv (lane 3), digested with the *Bst*BI and probed with [^{32}P]5′-end labelled anticodon oligonucleotide. *Hind*III digested λ-DNA was used as marker. (**C**) *Bst*BI and *Bam*HI digestion of 6·0 kb *Bam*HI clone. Photograph of ethidium bromide stained agarose gel and the corresponding Southern blot probed with [^{32}P] end labelled 5′ DNA oligomer are shown in lanes 1 and 2, respectively. The sizes of the various fragments are as indicated (also refer to figure 5). The 2·8 kb band corresponds to pTZ18R vector.

3.3 *Isolation and partial sequencing of the 6·0 kb BamHI fragment; and linkage of metA and metB loci*

From the above data it was not possible to deduce the linkage of the two *met* gene loci. Moreover, the results did not clarify whether or not there is a complete copy of IS*6110* at the initiator tRNA locus. To address these questions we screened a partial genomic library of *Bam*HI digested *M. tuberculosis* H37Ra DNA (5·5 to 7·0 kb fragments). Two clones having a 6·0 kb *Bam*HI insert (as indicated in figure 5) in opposite orientations were used for further characterization. Sequence analysis

Table 1. Examples of integrative elements carrying 3′ half of tRNA gene in the attachment site (*attP*).

Integrative elements	Host range	3′ half of tRNA genes associated with the *attP* site	Remarks
SLP	*Streptomyces* species	$tRNA^{tyr}$	17 kb conjugative plasmid (Reiter *et al.* 1989)
pMEA 100	*Nocardia mediterranei*	$tRNA^{phe}$	Transmissible plasmid (Reiter *et al* 1989)
pSAM 2	*Streptomyces* species	$tRNA^{pro}$	11 kb conjugative plasmid (Mazodier *et al* 1990)
HP1c1	*Haemophilus influenze*	$tRNA^{leu}$	Bacteriophage (Hauser *et al* 1992)
P4	*E. coli*	$tRNA^{leu}$	Bacteriophage (Pierson *et al* 1987)
Bacteriophage 16-3	*Rhizobium meliloti*	$tRNA^{pro}$	Bacteriophage (Papp *et al* 1993)
NBU1	*Bacteroides* species, *E. coli*	$tRNA^{leu}$	Nonreplicative, mobilized in *trans* by Tns (Shoemaker *et al* 1996)
SSV1	*Sulfolobus* strain B12	$tRNA^{arg}$	15·5 kb plasmid (Reiter *et al* 1989)

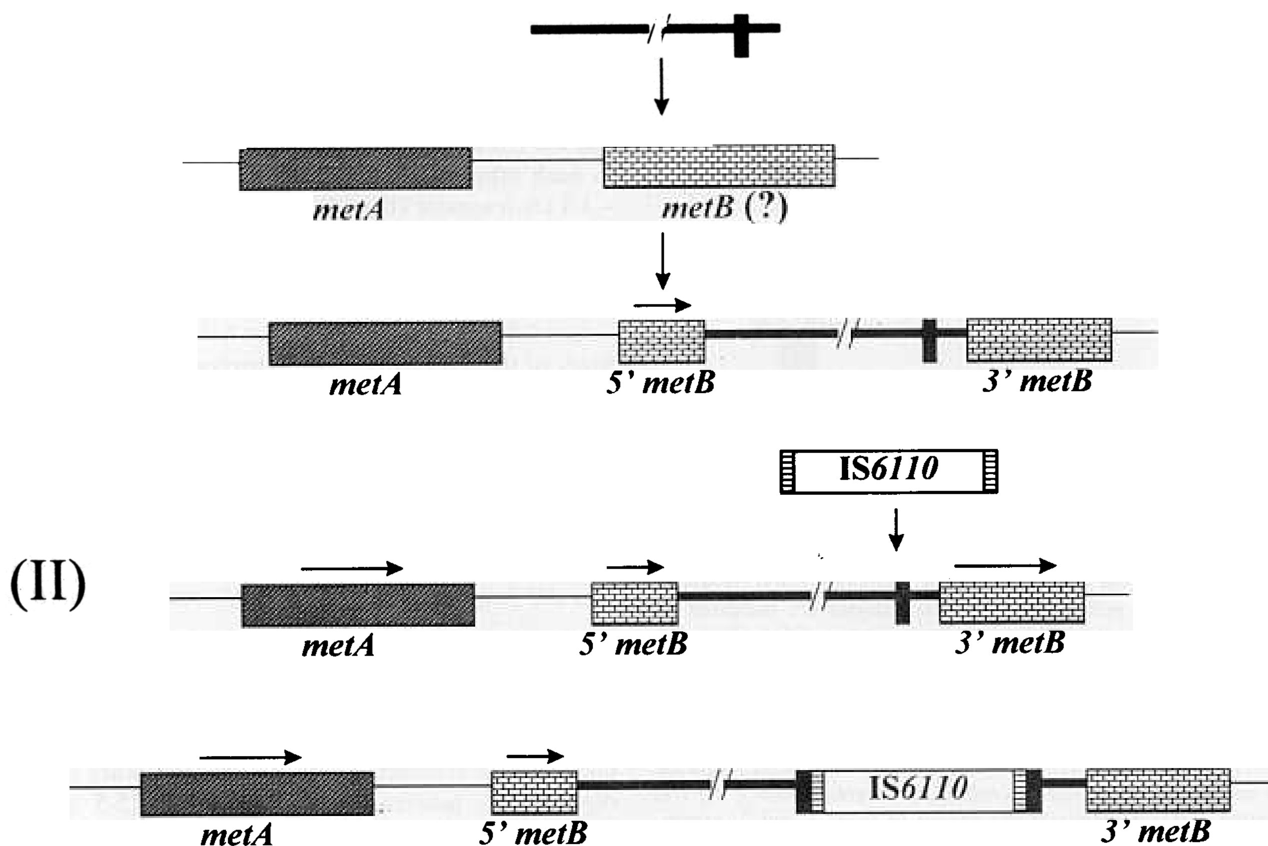

Figure 7A. For caption, see page No. 108.

revealed the presence of IS*6110*, corresponding to the sequence downstream of the *Bam*HI site (position 881, IS*6110* numbering) on one end, and *metA* sequence on the other. The identity of the upstream (5′-TGAACCGCCCCGGCATGTCCGGAGACTC-3′) and downstream (5′-GAGTCTCCGGACTCACCGGGGCGG-TTCA-3′) inverted repeats of IS*6110* flanked by direct repeats (ATT) was also confirmed (figure 5). These results are in agreement with the predictions from the data shown in figure 4 and unambiguously establish that the 1·7 kb and 6·0 kb *Bam*HI fragments are contiguous and, *metA* and *metB* are separated by IS*6110*.

3.4 *tRNA gene copy numbers in slow- and fast-growing mycobacteria*

The slow growing mycobacteria possess one operon for ribosomal RNA genes (*rrn*) as opposed to two in the fast growing mycobacteria (Clark-Curtis 1990; Ji *et al* 1994a,b). Our studies show that while *M. tuberculosis* H37Ra and H37Rv contain two initiator tRNA loci; only one of them, *metA*, represents an intact copy of the transcriptionally active gene. Contrary to the presence of two copies of ribosomal RNA genes, our results (figure 2) suggested a single copy of the initiator tRNA gene in *M. smegmatis* SN2. To establish the copy number in this fast growing mycobacteria, we performed a Southern blot analysis of *Bst*BI digested genomic DNA using the anticodon oligo as probe. The *Bst*BI site corresponds to TψC loop sequence region and is present in all the initiator tRNA genes. Southern blot analysis using anticodon oligo as probe ensures that the number of hybridizing bands in the *Bst*BI digested genomic blots exactly corresponds to the number of initiator tRNA genes in the genome. Consistent with our earlier obser-

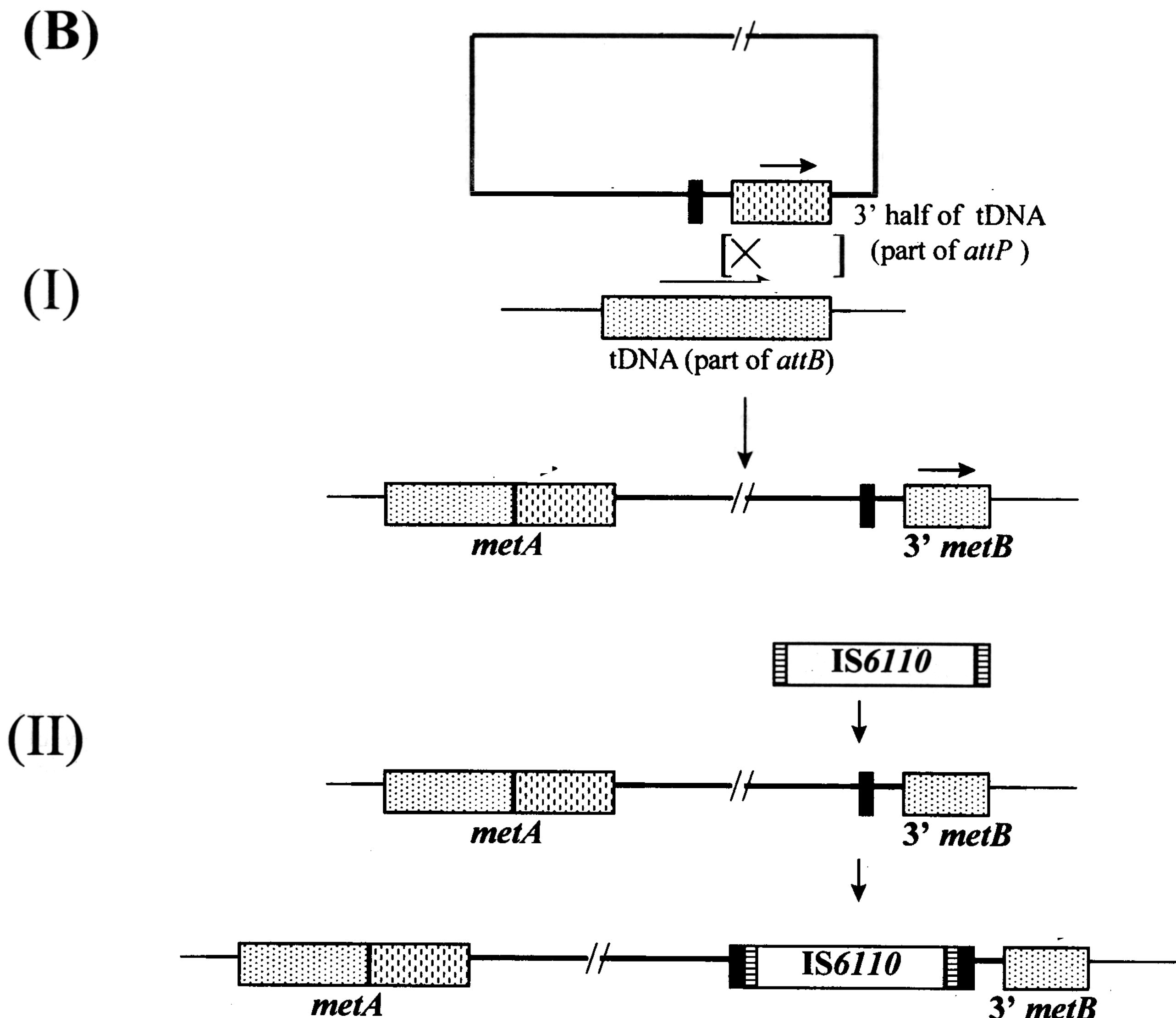

Figure 7B. For caption, see page No. 108.

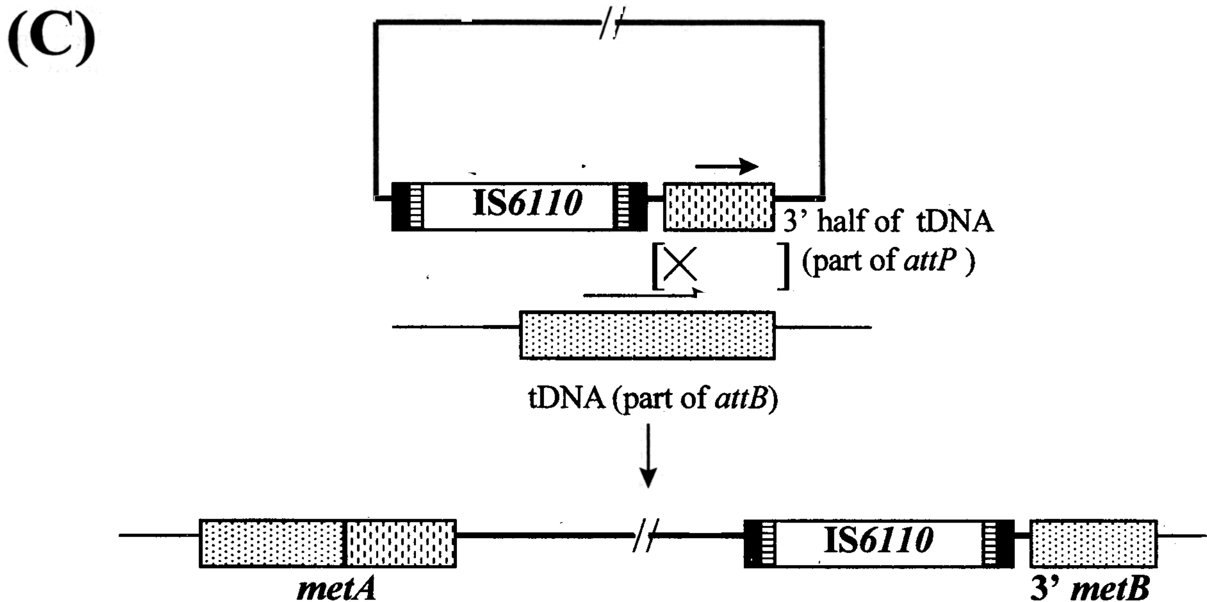

Figure 7. A schematic of the various models proposed to explain the origin of the initiator tRNA gene locus. (A) The shaded boxes show two copies of tRNA genes (*metA* and *metB*). *Step I*: disruption of *metB* by an insertion element (dark line with a break). *Step II*: Integration of IS*6110* into the first insertion sequence. (**B**) Site specific recombination between the integrative plasmid carrying the 3′ half of the initiator gene (as part of *attP*) and the initiator tRNA gene of the host (part of *attB*). The second event of insertion can then lead to integration of IS*6110*. (**C**) A variation of the model in (**B**). The integrative plasmid also carries the IS*6110* and hence a single recombination event results in the integration of 3′ half of *metB* and IS*6110*. For the sake of simplicity, the sites of recombination in (**B**) (step I) and (**C**) have been shown towards the left terminus of the 3′ half of the tDNA sequence of *attP*. However, recombination could occur anywhere within the tDNA sequence, as indicated by square brackets.

vation (figure 2), *M. smegmatis* showed a single band (lane 1, figure 6B) confirming the presence of a single initiator tRNA gene in this fast growing species. As expected, *M. tuberculosis* H37Ra and H37Rv show two identical size bands corresponding to *metA* and *metB* (lanes 2 and 3, figure 6B). Further, Southern blot analysis of *Bam*HI digested DNA using *metA* as probe detected two bands of 6·0 kb (harbouring *metA*) and 1·7 kb (harbouring *metB*) in both the strains (data not shown). Taken together, these results suggest an identical organization of *metA* and *metB* in *M. tuberculosis* H37Ra and H37Rv.

3.5 *Insertion sequences and the origin of metB*

Southern blots of *Bam*HI and *Bst*NI digested genomic DNA (figure 3) showed 9 to 12 clearly resolved bands. However, the contig map of *M. tuberculosis* H37Rv (Philipp *et al* 1996) revealed 16 copies of the IS*6110* in the genome thus suggesting that some of the bands in figure 3 may contain more than one copy of the IS*6110*. In any case, the *metB* locus represents the first example in mycobacteria where a mobile genetic element is seen to flank a tRNA-like sequence. There are a large number of reports where a tRNA loci in other eubacteria and archaebacteria have been shown to be the targets for site specific integration of various genetic elements (Inokuchi and Yamao 1995; Mazodier *et al* 1990; Papp *et al* 1993; Reiter *et al* 1989; Shoemaker *et al* 1996; Vogtli and Cohen 1992). However, as suggested by the presence of a 100 bp intervening stretch between the truncated tRNA-like sequence and IS*6110* beginning at position 783 (see figures 1B and 5), integration of IS*6110* at the *metB* locus is unlikely to be the primary event in the origin of the truncated tRNA-like sequence in *M. tuberculosis*.

A schematic diagram of the possible events which could have led to the present status of the initiator tRNA gene locus are shown in figure 7(A–C). *metB* could be a remnant of a previously active second copy of the initiator tRNA gene and evolved as a result of two insertion events. The first event disrupted the tRNA gene, and the second event led to the integration of IS*6110* into the first insertion sequence (figure 7A). This not only explains the presence of 100 bp sequence between *metB* and IS*6110*, but also predicts the presence of 5′ half of *metB* somewhere downstream of the insertion sequence(s). To test this model, we performed a Southern blot analysis on the *Bst*BI digest of the 6·0 kb *Bam*HI fragment, using an oligomeric probe corresponding to

the 5′-part of the initiator tRNA (figure 6A,C). In this experiment, we failed to detect a hybridizing signal which could correspond to the 5′ half of the *metB* tRNA. However, as expected, the 600 bp fragment harbouring the 5′ half of the *metA* gene was detected. While our failure to detect the 5′ half of the *metB* does not support this model, the interpretation is subject to the assumption that the 5′ half of *metB* did not diverge as a result of accumulation of mutations. Alternatively, it is likely that *metB* was introduced into the *M. tuberculosis* genome by an integrative plasmid. Many such plasmids carry the 3′ half of the tRNA gene as a part of the attachment site (*attP*). Although their integration into the genome displaces the 3′ half of the host tRNA gene (part of *attB*), they reconstitute a complete copy of the tRNA gene with the 3′ part coming from *attP* (table 1 and the references cited therein). As shown in figure 7(B), a second event of insertion of IS*6110* into the region next to the *metB* sequence could explain the origin of *metB* locus. Another likely possibility is that the integrative plasmid carried both the *metB* and IS*6110* element, and a single insertion event could have resulted in the present day initiator tRNA locus (figure 7C).

Further, many bacteriophages are also known to carry 3′ half of a tRNA gene as a part of *attP*. Thus a variation of the models shown in figure 7(B,C) could be that *metB* came as part of a temperate phage (Reiter *et al* 1989 and the references cited therein; Papp *et al* 1993). Since, the distance between *metA* and *metB* is only ~ 6·0 kb, we consider the insertion of a temperate phage (average size ~ 40–50 kb) an unlikely possibility. To gain further insight into the mechanism of origin of *metB*, we performed homology search of sequences neighboring *metB*. However, the sequence revealed no homology to any known insertion elements, integrative plasmids or bacteriophages. Nevertheless, the question remains, 'Is *metA* the original initiator tRNA gene?'

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