# Synopsis

Protein synthesis in all organisms utilizes a special tRNA called the initiator tRNA. Initiator tRNAs take part in the initiation step of protein synthesis by their direct binding to the P-site of the ribosome. The other tRNAs (elongator tRNAs) bind first to the A-site of the ribosome and are subsequently translocated to the P-site during elongation. The initiator tRNA possesses sequence and structural characteristics, which enable it to perform its unique function in protein synthesis. In addition to the highly conserved three consecutive G:C base pairs in the anticodon stem of the initiator tRNA which facilitate its P-site binding, bacterial and organellar initiator tRNAs are also formylated by FMT (methionyl-$tRNA^{fMet}$ formyltransferase) to enable their binding to initiation factor 2 (IF2), directing them specifically into initiation. Structure-function studies of *E. coli* initiator tRNA *in-vivo* using reporter constructs showed that formylation plays a crucial role in deciding the fate of the initiator tRNA in initiation. The tRNA mutants deficient in formylation take part in initiation and/or elongation. Protein factors like IF2, elongation factor Tu (EF-Tu) and peptidyl-tRNA hydrolase (Pth) also contribute to the fate of the tRNA *in-vivo*. The current study aims to understand how the balance of protein factors and sequence elements present on a tRNA determine its participation at the steps of initiation and/or elongation using *E. coli* and *M. smegmatis* as model organisms. The findings of my research have been described in three distinct investigations as follows:

**PART-I. Development of assay systems for amber codon decoding at the steps of initiation and elongation by $tRNA^{fMet}$ derivatives in mycobacteria**

The bulk of our understanding of the mechanism of protein synthesis in bacteria is derived from the studies in *E. coli*. The mechanism of translation in Gram positive bacteria remains a relatively less understood process. Gram positive bacteria possess significant differences in their translational apparatus as compared to the Gram negative organisms, and therefore present with interesting systems to understand the mechanism of translation. For example, Gram positive bacteria use an indirect pathway for synthesis of Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$ as opposed to direct synthesis of Gln-$tRNA^{Gln}$ by glutaminyl-tRNA synthetase (GlnRS) in *E. coli*.

We used *M. smegmatis*, a slow growing Gram positive bacterium, as the model organism to study translation. The understanding of protein synthesis in these bacteria has been limited by the lack of well characterized genetic systems. Using chloramphenicol acetyltransferase (CAT) reporters (having an amber codon as the start codon or as a codon at an internal position within the reading frame of the mRNA), we developed genetic systems where the amber codon is decoded by a mutant initiator tRNA (wherein the CAU anticodon was mutated to CUA with or without additional changes in the acceptor stem) either at the step of initiation or elongation in *M. smegmatis*, enabling us to measure the efficiency of the mutant tRNAs in initiation or elongation *in-vivo*. Characterization of the reporter encoded protein by mass spectrometric analysis showed that initiation in such a reporter proceeds through incorporation of methionine, as opposed to the use of glutamine in similar systems in *E. coli*. Elongation in the reporter system, carried out by formylation deficient mutants of the initiator tRNA, was found to occur through the insertion of glutamine using a two-step pathway, where the tRNA is first aminoacylated by a non-discriminating GluRS, followed by transamidation of the attached Glu by an amidotransferase. The formylation deficient acceptor stem mutants of the initiator tRNA were also recognized differentially by the amidotransferase *in-vivo*, leading to the insertion of either glutamate or glutamine during elongation by different mutants of the tRNA. Overall, the study highlighted the conserved nature of formylation across bacteria, and its importance in the exclusive participation of the initiator tRNA in initiation.

**PART II. Physiological role of tRNAs that function as alternate initiator tRNAs in mycobacteria**

The genomes of *M. tuberculosis* and *M. smegmatis* encode three tRNAs, *metU*, *metV* and *metT*, with CAU anticodons. While *metU* has been shown to encode the initiator tRNA in these bacteria, the other two (*metV* and *metT*) are both annotated as methionine elongator tRNA. Interestingly, these tRNAs also possess some sequence characteristics like the G:C base pairs in the anticodon stem, and the lack of a Watson-Crick base pair at 1:72 position, which are thought to be restricted to initiator tRNAs. We were interested in understanding the physiological role of such sequence elements present in the elongator tRNA. Computational and biochemical characterization of *metT* and *metV* identified them as the methionine decoding elongator tRNA ($tRNA^{Met}$) and the minor form of the isoleucine decoding tRNA

($tRNA^{Ile2}$), respectively. Mass spectrometric analysis of *metV* showed that the C34 in the anticodon of the tRNA is modified to lysidine, which is consistent with its role in AUA decoding. Interestingly, the expression of *metV* is upregulated under stress conditions. Analysis of the modification status of the tRNA under hypoxic conditions showed that the tRNA is undermodified for lysidine, which would preclude its function in AUA decoding and enable it to function as $tRNA^{Met}$. We propose that this could be a mechanism of regulation employed by cells under hypoxia. *In-vivo* assays using the CAT reporter showed that *metV* is capable of initiating protein synthesis, providing support to our hypothesis that such tRNAs could function as alternate initiator tRNAs under stress conditions.

**PART III. Mitochondria-like sustenance of *E. coli* on a single $tRNA^{fMet}$**

Most organisms possess distinct methionine tRNAs that participate at the steps of initiation and elongation. However, protein synthesis in mammalian mitochondria utilizes only a single $tRNA^{Met}$, which functions in both initiation and elongation. The partitioning of tRNA into initiation and elongation phases is thought to occur due to the competition between $EF\text{-}Tu_{mt}$ (which directs the tRNA to the elongation step) and $Fmt_{mt}$ (which, following formylation, directs the tRNA to the initiation step), for their binding to the single species of $Met\text{-}tRNA^{Met}$.

Our studies on initiator tRNA from mycobacteria, along with the available literature on mutants of *E. coli* initiator tRNA, show that some mutants of initiator tRNA are capable of participating at the step of elongation. Similarly, as shown in Part-II of the results section, native elongator tRNAs capable of initiating protein synthesis also exist in some organisms. We therefore asked if in *E. coli*, a single $tRNA^{Met}$ could participate at both the steps of initiation and elongation (as in mitochondria), and if such tRNAs fulfill the cellular needs at both steps. Using a transduction based genetic strategy, we have shown that many acceptor stem mutants of the initiator tRNA sustain *E. coli* for initiation and elongation function separately. Importantly, a subset of these mutants also sustains the growth of *E. coli* devoid of all the six copies of methionine tRNA genes (four of initiators and two of elongators). Furthermore, the mutant tRNA which is most efficient at sustaining the cell is also the one which is most like mitochondrial $tRNA^{Met}$. Overall, the study provides insights into how distribution of the tRNA between FMT/IF2 and EF-Tu determines its role in protein synthesis.