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Review Article

Role of The Two Highly Conserved Features of Initiator tRNAs in Initiation of Protein Synthesis in Eubacteria

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Regulation of translation represents a crucial step in gene expression. In this mini-review, we provide a brief outline of protein synthesis in bacteria, and discuss the participation of a special tRNA, the initiator tRNA (i-tRNA), in the different stages of the initiation step. The conserved features of the bacterial i-tRNA are located in its acceptor and anticodon stems. A Watson-Crick mismatch pair (1x72) at the top of the acceptor stem along with the 2:71 and 3:70 pairs, is important in formylation of the amino acid attached to the i-tRNA. The property of formylation facilitates i-tRNA binding to the 30S ribosome. The second feature of the presence of the three consecutive G:C pairs (29:41; 30:40 and 31:39 positions) in the anticodon stem, highly conserved in the i-tRNAs in all domains of life, plays a crucial role in the transition of i-tRNA through the different stages in formation of the 70S elongation competent complex from the 30S pre-initiation complex.

Keywords: Initiator tRNAs; Protein Synthesis

Introduction

Translation of mRNA into protein, a major hub for regulation of gene expression, comprises initiation, elongation, termination and recycling steps and is catalyzed by large ribonucleoprotein complexes called ribosomes. Bacterial ribosomes comprise a large (~1.5 MDa; 50S) and a small (~0.85 MDa; 30S) subunits made of ribosomal proteins (r-proteins) and RNA (rRNA) which together constitute 70S ribosome with E-, P- and A- sites for binding transfer RNAs (tRNAs). The tRNAs facilitate the process of translation by serving as adaptor molecules that bring amino acids (aa) to the ribosome in response to the array of the triplet nucleotides (codon) in the messenger RNA (mRNA). Precise reading of the codons in mRNA that begins with the identification and recognition of the first codon, the start or the initiation codon (during the step of initiation) is crucial for production of a meaningful protein (Laursen *et al.*, 2005). Thus, cells possess two kinds of tRNAs, an initiator tRNA (i-tRNA, a special tRNA which binds directly in the P-site to read the start codon) and the elongator tRNAs (that bind in the A-site to decode the subsequent codons). Selection of the correct aa-tRNA in the A-site of the ribosome is a key process that ensures fidelity of translation. Ribosomes catalyze repetitive reactions of transferring the aa or the peptide from the P-site tRNA to the aa on the A-site tRNA by peptide bond formation during elongation (Fischer *et al.*, 2010). The A-site tRNA (now attached with a peptide) is moved to the P-site and, the deacylated P-site tRNA is simultaneously moved to the E (exit)-site together with the concerted movement of mRNA (translocation), exposing the next codon in A-site to bind another aa-tRNA. The peptide bond formation results in location of the tRNAs in the hybrid states, the P-site tRNA originally in the P/P state (P-site of 30S and P-site of 50S) transitions into the P/E state (P-site of 30S and E-site of 50S), and the A-site tRNA bound in the A/A state, into the A/P state together with the anticlockwise movement (ratcheting) of the 30S subunit, resulting in a rotated state of the ribosome. The process of translocation is crucial to convert the

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rotated ribosomes back to the nonrotated state and to transfer the P/E and A/P hybrid state tRNAs into the E/E and P/P states to expose a new codon in the A-site (Dunkle *et al.*, 2011; Fischer *et al.*, 2010). Arrival of a special codon (termination codon) at the end of mRNA reading frame in the A-site, prompts the release of the synthesized polypeptide, and dissociation of the ribosomal subunits from the mRNA to begin a new round of protein synthesis. Numerous protein factors assist the energy consuming process of protein synthesis. Initiation factors (IF1, IF2 and IF3) play crucial roles during initiation; elongation factors EFTu and EFG help recruitment and movement (translocation) of tRNAs, respectively; release factors (RF1, RF2 and RF3) facilitate release of the newly synthesized polypeptide; and the ribosome recycling factor (RRF) together with EFG and IF3 separates the ribosomal subunits from the mRNA (Schmeing and Ramakrishnan, 2009).

Translation initiation is a rate limiting step in protein synthesis and subject to various regulatory mechanisms in all kingdoms of life (Gold, 1988). The canonical pathway of initiation of protein synthesis in bacteria makes use of the three highly conserved and essential initiation factors (IF1, IF2 and IF3); and, binding of mRNA to the 30S subunit is facilitated by the interaction of a purine rich sequence (Shine Dalgarno sequence, SD) in mRNA (occurring upstream of the start codon) with a sequence towards the end of 16S rRNA in the 30S subunit. The i-tRNA binds in the P-site and its anticodon pairs with the start codon in the mRNA. The initiation factors, particularly IF3 ensures fidelity of i-tRNA binding in the P-site and recognition of the start codon. The complex of 30S ribosome with i-tRNA, mRNA, and the initiation factors is designated as 30S pre-initiation complex (30S PIC), which is then joined by the 50S subunit to form a 70S complex, that is finally converted into an elongation competant 70S complex (70S EC) upon the release of the initiation factors. The 70S EC possesses the i-tRNA bound in the P-sites of both the 30S and 50S subunits (P/P state) and has the A-site available to bind to the aa-tRNA specified by the mRNA codon in the A-site (Figs. 1, 2).

Recent single molecule studies show that there can be multiple pathways to reach the formation of 30S PIC (Tsai *et al.*, 2012). In cryo-electron microscopic structures of 30S PIC, it was observed that i-tRNA is not found in the P/P state as in the 70S EC, instead it is slightly tilted towards E-site of the ribosomes (P/I state) (Julian *et al.*, 2011). Further, loading of the large ribosomal subunit (50S) might happen in this stage only. Single molecule studies have also suggested that hydrolysis of IF2 bound GTP upon 50S subunit binding causes rotational movement (opposite to the usual ratcheting occuring during the elongation step) of the two subunits in such a way that i-tRNA is now in P/P state. This in turn, triggers

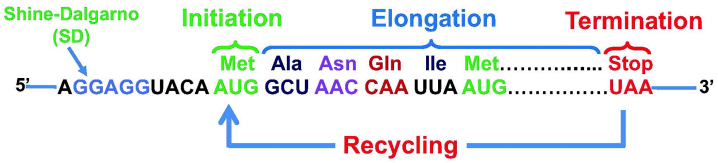

Fig. 1: Schematic of translation of mRNA

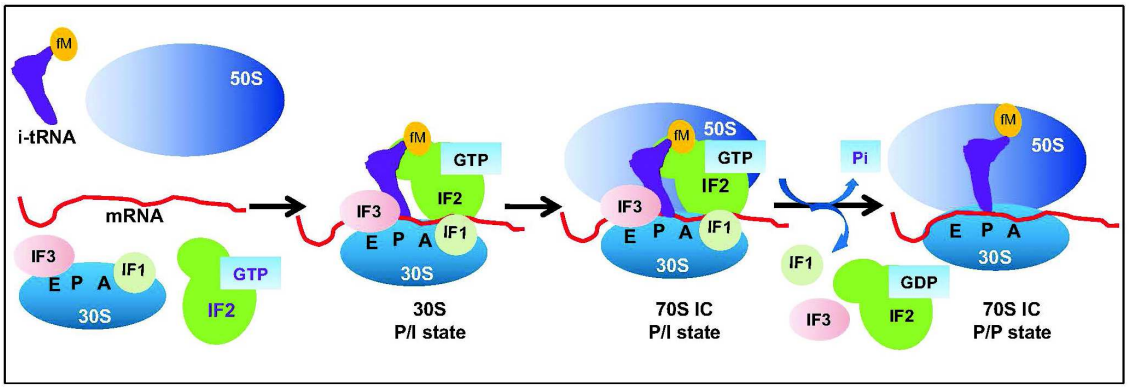

Fig. 2: Various stages in translation initiation in eubacteria

the release of IF1, IF2, as well as IF3 to produce 70S EC (Marshall *et al.*, 2009; Tsai *et al.*, 2012). Thus, initiation process possesses distinct stages defined by many conformational changes.

Our research activities have focused on the process of selection of the i-tRNA in the P-site and its participation through the multiple stages in initiation. Thus, i-tRNA provides us with an excellent probe to understand the mechanism of initiation. Here, we discuss how the salient features of i-tRNA contribute to the various reactions in the process of initiation, primarily based on our own findings.

Initiator tRNA and Its Unique Features

E. coli chromosome possesses four copies of i-tRNA genes. Three of them (*metZWV*) are located at 63.5 min, whereas the fourth one (*metY*) is located some distance away at 71.5 min. Selection of i-tRNA (Fig. 2) in the P-site of the ribosome is primarily assisted by its unique features and the various IFs (RajBhandary, 1994). The two important features of i-tRNAs are, (a) the presence of a Watson-Crick mismatch pair at the 1x72 position in the acceptor stem, and, (b) the presence of three consecutive G:C base pairs (GC/GC/GC, 3GC pairs) at 29:41, 30:40, 31:39 positions in the anticodon stem of i-tRNA (Fig. 3; (Mandal *et al.*, 1996; Marck and Grosjean, 2002)). IF2 has much higher affinity for the formyl-aa-tRNA and facilitates binding of i-tRNA to the P-site (Mangroo and Raj Bhandary, 1995; Wu and Raj Bhandary, 1997). IF3, an anti-association factor in *E. coli*, has a major role in selection of i-tRNAs (Antoun *et al.*, 2006a; Antoun *et al.*, 2006b; Karimi *et al.*, 1999). IF1 has also been proposed to enhance initiation by favoring binding of IF2 (Antoun *et al.*, 2006a; Antoun *et al.*, 2006b). Thus, all the three initiation factors make the ribosomal P-site selective for i-tRNA binding. In addition, the tails of the r-proteins S9 and S13 and the highly conserved 16S rRNA nucleotides, G1338 and A1339, in the P-site have been shown to be crucial in the selection of i-tRNA in the P-site (Arora *et al.*, 2013a; Arora *et al.*, 2013b; Lancaster and Noller, 2005; Selmer *et al.*, 2006).

Mismatch at the 1x72 Position of the i-tRNA and the Role of Formylation

The mismatch at the top of the acceptor stem (position 1:72) is conserved in i-tRNAs from eubacteria (C1xA72 in *E. coli*) and is not found in the elongator tRNAs. This mismatched pair contributes to several of the required properties of the i-tRNAs and provides a crucial determinant for formylation of the aa attached to i-tRNA, by Fmt (10-Formyl tetrahydrofolate methionine transferase or formylase) (Guillon *et al.*, 1992b; Lee *et al.*, 1992; Lee *et al.*, 1991). The formylation reaction increases the affinity of i-tRNA to IF2 (Sundari *et al.*, 1976). An efficient rate of formylation of the aminoacylated i-tRNA also avoids its partitioning with EFTu, and its participation in elongation (Nissen *et al.*, 1995; Seong and RajBhandary, 1987b). Single molecule studies suggested that formylation enhances the formation of 30S initiation complex and the stability of the 70S complex (Ling and Ermolenko, 2015; Masuda *et al.*, 2012). Although the lack of Fmt causes severe growth retardation of bacteria, it is not absolutely essential in *E. coli* under the laboratory culture conditions (Guillon *et al.*, 1992a). Interestingly, the growth defect in bacteria occuring due to the lack of Fmt is rescued by the amplification of gene copy numbers in the i-tRNA loci (Nilsson *et al.*, 2006). Likewise, overexpression of i-tRNA from plasmid borne i-tRNA genes also rescues for the deficiency of formylation (Shetty *et al.*, 2017). These observations suggest that the formylation of aminoacylated i-tRNA primarily supports at some of the very early steps, such as its initial recruitment to the 30S ribosome. The high copy number of i-tRNA genes in most organisms (4 in *E. coli*) also contributes to prevention of elongator tRNA binding to the P-site (Kapoor *et al.*, 2011). Thus, formylation is crucial not only for the higher efficiency of initiation but also for the high fidelity of initiation. In case of eukaryotes, there is no formylation of aa attached to the i-tRNA. However, the A1:U72 base pair enhances its binding to eIF2-GTP (Farruggio *et al.*, 1996; Kapp and Lorsch, 2004) as well as the binding of the ternary complex (aminoacylated i-tRNA, eIF2-GTP) to 40S pre-initiation complex (Kapp *et al.*, 2006). It is also needed for survival of yeast cells (Astrom *et al.*, 1993; von Pawel-Rammingen *et al.*, 1992).

Role of the 3GC Base Pairs

Unlike formylation, which is conserved only in eubacteria, the presence of 3GC base pairs in the anticodon stems of i-tRNAs is highly conserved in all domains of life (barring some exceptions), suggesting

a critical role for this feature in the process of initiation. In fact, using both *in vivo* and *in vitro* experiments, it has been shown that the lack of the 3GC pairs renders the i-tRNA mutants inefficient in translation initiation or in their stable binding to ribosomes (Mandal *et al.*, 1996; Seong and RajBhandary, 1987a; Varshney *et al.*, 1991). Although the precise role of the 3GC pairs has remained unclear, in the crystal structures of 70S ribosomes containing mRNA and i-tRNA in the P-site, the two universally conserved residues G1338 and A1339 of 16S rRNA (G1575 and A1576 of 18S rRNA in yeast) are poised to make A-minor interactions with the minor groove sides of the first and the second G:C base pairs (Korostelev *et al.*, 2006; Selmer *et al.*, 2006). *In vitro* studies showed that these interactions stabilize the binding of i-tRNA in the ribosomal P-site (Lancaster and Noller, 2005; Qin *et al.*, 2007).

Naturally Occurring Exceptions to the '3GC Rule' and the Minimalist '3GC Rule'

We observed that at least some of the mycoplasmal and rhizobial species possess variations in the 3GC pairs of i-tRNAs (Fig. 3). These natural variants possess either AU in place of the first GC (1st AU, or

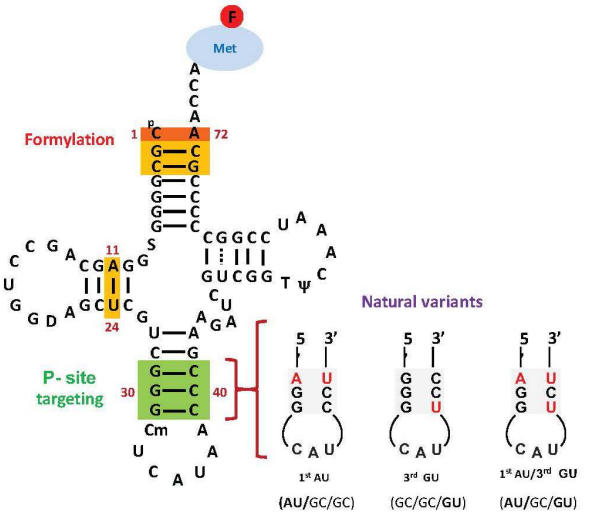

Fig. 3: Sequence and clover leaf structure of initiator tRNA and its naturally occurring anti-codon stem variants. The 3GC base pairs are highlighted in green, the formylation features are highlighted in yellow/ orange. The presence of a mismatch at the 1x72 position in the acceptor stem is shown highlighted in orange. Mycoplasmal and rhizobial natural variants of initiator tRNAs are shown highlighted in grey and labelled according to the variation present

AU/GC/GC), GU in place of the third GC (3rd GU, GC/GC/**GU**) or both (**AU**/GC/**GU**) (Samhita *et al.*, 2012); (Fig. 3). These observations raise a question on the essentiality of the occurrence of the 3GC pairs. Thus, to understand the importance of the 3GC pairs and to investigate if these (mycoplasmal and rhizobial) unconventional i-tRNAs could function in *E. coli*, we used the approach of deleting all the four i-tRNA genes from *E. coli* genome in the presence of plasmid borne copies of the unconventional i-tRNA genes. As the presence of functional i-tRNA is essential for *E. coli* growth, deletion of all the i-tRNA genes (*metZWV* and *metY*) would materialize only if the variant i-tRNAs (plasmid borne) were functional in *E. coli*. The i-tRNAs with naturally occurring variants of the 3GC pairs sustained *E. coli* for the deletion of *metZWV* and *metY*. However, those that contained either the sequence corresponding to that of the elongator $tRNA^{Met}$ (i.e. **UA**/**CG**/**AU**) or GC/**CG**/**AU** or GC/**CG**/GC failed to do so (Samhita *et al.*, 2012). Interestingly, even though the naturally occurring variants of i-tRNAs which sustained *E. coli* growth did not possess 3GC pairs, they still possessed a sequence with 3(Purine:Pyrimidine) pairs. This observation raised the possibility of requirement of three Purine:Pyrimidine base pairs rather than the 3GC pairs as the essential feature for the i-tRNA function. Extensive mutagenesis of individual GC pairs into other base pairs such as AU and CG pairs suggested that among the three base pair positions, first and third pairs could be changed to AU or CG pairs, thus requirement of three Purine:Pyrimidine pairs was also ruled out. However, the mutants containing either AU or CG pair in place of the middle GC pair did not sustain *E. coli*. This implied that middle GC pair is essential for i-tRNA function (Fig. 4; (Shetty *et al.*, 2017).The mutants containing AU, CG or GU in the 1st or the 3rd base pair positions in different combinations sustained *E. coli* in the context of middle GC pair.

The i-tRNA mutant with all its 3GC pairs replaced with AU pairs (**AU**/**AU**/**AU**) did not sustain *E. coli* for its growth. However, conversion of middle AU to GU (by mutating A30 back to G30) in the **AU**/**GU**/**AU** mutant allowed it to sustain *E. coli* growth revealing that the role of G30 is the most crucial. Although it should also be said that the middle GU pair did not support *E. coli* growth when it occurred in the context of the flanking pairs as CG (i.e. **CG**/

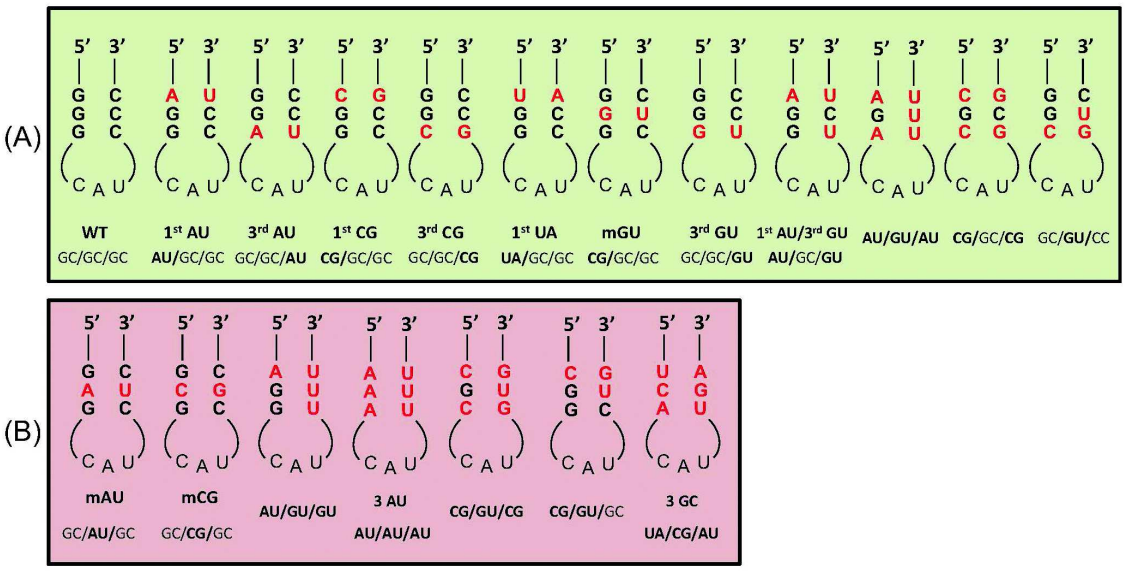

Fig. 4: Anticodon stem loop of wild type and mutant initiator tRNAs. Wild type initiator tRNA anticodon stem containing full complement of 3GC base pairs is shown as WT, mutations in the 3GC base pairs are shown in red and labelled according to the mutation. The mutants of 3GC base pairs that sustain *E. coli* lacking all the four copies of genomically encoded i-tRNA are shown in the green box, while mutants which could not sustain the cell are shown in the red box

GU/**CG**). Moreover, while the GC/**GU**/**CG** mutant supported *E. coli* growth, the **CG**/**GU**/GC did not, suggesting that the middle GU pair can function only in the context of a Purine:Pyrimidine pair at the 1stGC position, i.e. either GC or AU pair. These observations suggest that presence of not just G30 but also the right structural context for it are crucial. Structural and molecular dynamic analyses might facilitate further understanding on the presentation of the G30 residue in the correct structural context. It may also be noted that the U residues in positions 38, 39 and 40 could be pseudouridylated by TruA (Hur and Stroud, 2007) and it would be of interest to investigate what role(s) such a modification might have.

Another question that arises from our mutational analyses is that if the middle GC or even G30 in an appropriate context are adequate to sustain *E. coli*, why the 3GC pairs have been so highly conserved in all domains of life. Importantly, when we analysed relative growth of *E. coli* strains sustained on different i-tRNA mutants, we noted differences in growth especially at cold temperature as well as on minimal media, suggesting that the presence of a full complement of the three GC base pairs confers a fitness advantage for growth under the conditions of stress (Shetty *et al.*, 2017). Further, many elongator tRNAs do possess the middle GC pair (together with the 1st or the 3rd GC pair) in their anticodon stems. Thus, it would be of interest to see how such elongator tRNAs would compete with the i-tRNA, perhaps in the conditions of stress.

Importance of the 3GC pairs has also been investigated for yeast i-tRNA by similar approach of sustainability using plasmid encoded mutant i-tRNAs (Dong *et al.*, 2014; von Pawel-Rammingen *et al.*, 1992). Yeast provides an interesting system since in this organism both the initiator and elongator species of tRNA^{Met} possess the middle GC pair. Mutations in the 1st and the 3rd GC pairs revealed that changing GC pairs into even U:U pairs did not hamper the ability of the i-tRNA mutant to sustain yeast (von Pawel-Rammingen *et al.*, 1992). However, the *in vitro* reconstituted experiments suggested that the GC pairs are important for formation of 40S IC (Kapp *et al.*, 2006). Further, it was shown that the 3GC pairs are essential in yeast (Dong *et al.*, 2014). The study showed that neither of the 3 positions tolerated Purine:Purine pairs. Also, neither the 1st nor the 2nd GC pairs tolerated Pyrimidine:Pyrimidne pairs. However, the 3rd GC could be changed to Pyrimidine:Pyrimidine pairs to still retain the biological function of the mutant. Interestingly, in contrast to *E. coli*, in yeast i-tRNA, substitution of the middle GC pair with either AU or CG pairs still retained its

biological function to sustain yeast. In eukaryotes, scanning mode of initiation occurs where the 40S IC containing i-tRNA scans the 5' UTR and recognizes the start codon (Hinnebusch and Lorsch, 2012). During scanning, the 40S complex is in open conformation, while upon recognition of the start codon it attains closed conformation. As the process of initiation in yeast is mechanistically different, the exact requirement of the GC pairs might also be different. Extensive mutagenesis of the 3GC pairs in i-tRNA would be necessary to infer their precise role in yeast.

Role of the 3GC Pairs in Formation of 70S Complex

In vitro studies suggested that the lack of the 3GC pairs affects the stability of 30S IC (Hartz *et al.*, 1989). The *in vivo* reporter assays suggested that the 3GC pairs are crucial for translation initiation (Mandal *et al.*, 1996). Further, the *in vivo* analysis of abundance of i-tRNA across the polysome profiles showed that most of the wild type or the mutant i-tRNAs that support *E. coli* growth accumulate in the 70S ribosome population (with a much less of the i-tRNA fraction occurring in the 30S population). However, for an i-tRNA mutant unable to support *E. coli* growth (e. g. the **UA**/**CG**/**AU** mutant), most of the tRNA was found in the 30S population and very little in the 70S (Shetty *et al.*, 2014). These observations suggested that the 3GC pairs are crucial for transition of the i-tRNA from the 30S to 70S complexes. Also, unlike the rescue of the growth defect of *E. coli* due to the lack of the formylation of i-tRNA by increasing the amount of i-tRNA in the cells, overexpression of 3GC mutant i-tRNAs did not mitigate the growth defect of the cells harboring i-tRNAs defective in initiation due to mutation(s) in the 3GC sequence (Shetty *et al.*, 2017). These observations suggested that while the major role of formylation is in the initial binding of i-tRNA to the ribosome, that of the 3GC pairs is in the later stages of initiation. Thus, we proposed sequential roles of these two unique features, formylation being crucial in the recruitment of i-tRNA to the P-site, and the 3GC pairs governing the later steps of transition into the 70S EC (Fig. 5). Further, a genetic suppressor analysis suggested that the presence of an extended SD context in them RNA facilitated the availability of the 3GC mutant i-tRNA into the 70S complex (Shetty *et al.*, 2014).

Analyses with many other i-tRNAs mutated in their 3GC pairs suggested that while the 1st and the 3rd GC pair position mutants enter the 70S complex efficiently, the mid AU mutants showed a major defect in their transition to 70S (Shetty *et al.*, 2017). Interestingly,while the 3AU (**AU**/**AU**/**AU**) mutant failed to enter the 70S complex efficiently, re-introduction of G at position 30 (**AU**/**GU**/**AU**) allowed the i-tRNA to enter 70S complex suggesting an essential role of the G30 in the mid GC pair in the formation of the 70S complex. Surprisingly, while the midCG mutant is capable of entering the 70S complex efficiently, it fails to sustain *E. coli* for its growth. This observation prompted us to investigate for the additional roles of the 3GC pairs.

The 3GC Base Pairs and IF3 Release

Among the three initiation factors, IF3 plays a crucial role in the fidelity of initiation. Mutations in IF3 have been shown to affect 3GC pair discrimination as well as start codon selection (Ayyub *et al.*, 2017; O'Connor *et al.*, 2001; Petrelli *et al.*, 2003; Petrelli *et al.*, 2001). *In vitro* studies had suggested that IF3 destabilises elongator tRNA containing 70S IC (Antoun *et al.*, 2006a; Antoun *et al.*, 2006b). Further, it has been shown that IF3 discriminates the 3GC pairs by causing structural rearrangements in the 16S rRNA loop containning G1338, A1339 (Lancaster and Noller, 2005). It has also been proposed that in the presence of IF3, G1338 and A1339 residues allow only tRNA with the 3GC pairs to form initiation complex. Interestingly, when we analysed the 70S complexes from *E. coli* (supported by the chromosomal copy of i-tRNA gene) harboring midCG mutants, they retained more IF3 in 70S complex compared to the 70S complexes formed with the wild type or the i-tRNA mutants that support *E. coli* for its growth (Shetty *et al.*, 2017). Thus, although the mid CG i-tRNA mutant is able to transit into the 70S complex, it is defective in releasing IF3 from 70S, and thus in sustaining *E. coli* for its growth.

Conclusions and Future Perspectives

The two most distinctive elements of eubacterial i-tRNAs are located in their acceptor and the anticodon stems (RajBhandary, 1994; Varshney and Raj Bhandary, 1992). The mismatched base pair at the top of the acceptor stem (1x72) along with the 2:71 and 3:70 pairs in the acceptor stem are responsible

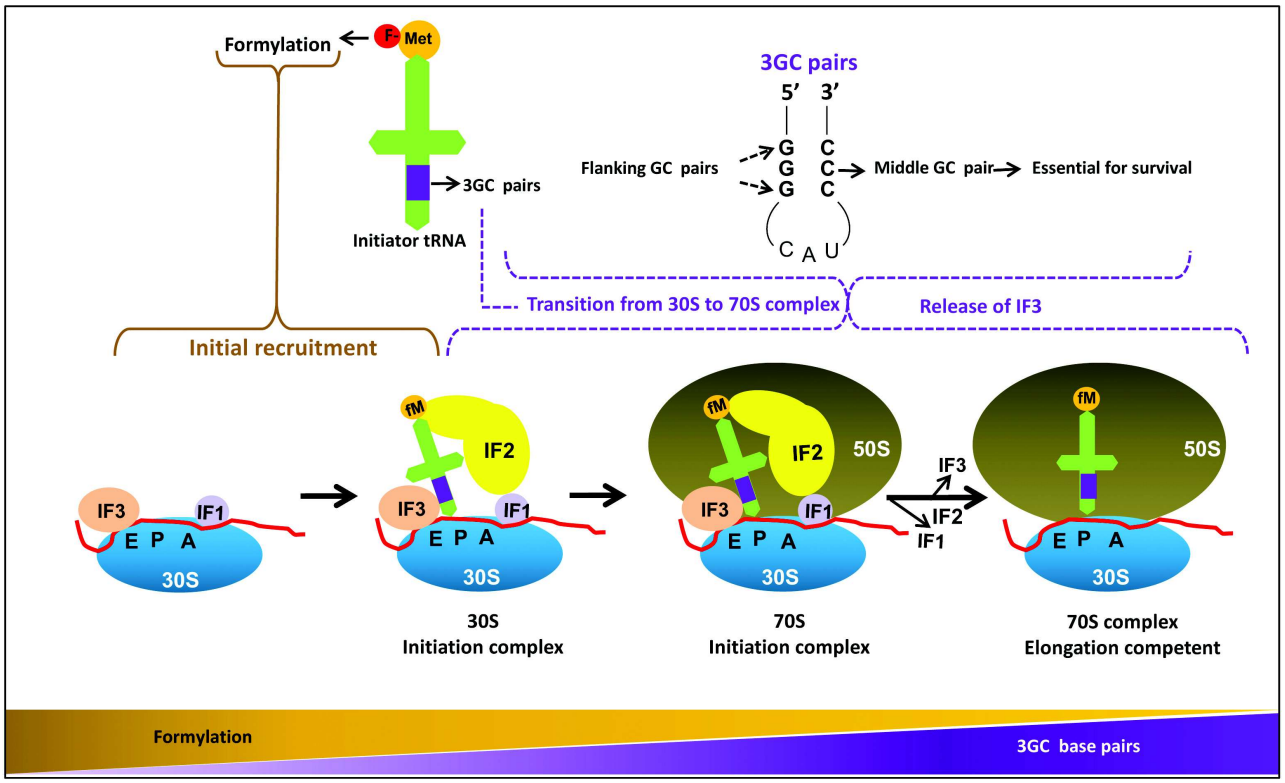

Fig. 5: Model showing the major roles of formylation and 3GC base pairs of initiator tRNA at different stages of initiation

for the recognition of an i-tRNA by Fmt for the formylation of the attached aa. The function of formylation of i-tRNA is primarily to facilitate its binding to IF2 and to the 30S ribosome. The second feature of the presence of the three consecutive G:C base pairs (3GC pairs) in the anticodon stem is highly conserved in all domains of life. In our earlier studies, we showed that incorporation of these two distinctive features of i-tRNAs into an elongator tRNA conferred upon it the initiation activity (Varshney *et al.*, 1993). In our recent studies, we have shown that the feature of the 3GC pairs, facilitates transition of the i-tRNA from the 30S ribosome to the 70S ribosome. In addition, we observed that the 3GC base pairs also facilitate release of IF3 from the 70S PIC (Fig. 5; Shetty *et al.*, 2017). More recently, we observed that the pioneering round of initiation is also responsible to prompt ultimate steps of the ribosome maturation by signaling RNases to trim the terminal extensions of immature 16S rRNA (Shetty and Varshney, 2016). The cellular level of i-tRNAs are subject to regulation under stress (ArgR, ppGpp, VapC) (Krin *et al.*, 2003; Nagase *et al.*, 1988; Winther and Gerdes, 2011). Does the deficiency of i-tRNA in cells lead to generation of ribosomal heterogeneity due to deficiency in the processing of the 16S rRNA at the 5'-, and the 3'-ends? Do the ribosomes with heterogenous ends influence translation of cellular mRNAs (e. g. by impacting SD anti-SD interaction)? Interestingly, it has also been shown that a slight deficiency of i-tRNA in *E. coli* confers fitness advantage for growth under nutrient limiting conditions (Samhita *et al.*, 2014). Investigation of how SD and anti-SD interactions impact the requirement of the 3GC pairs in i-tRNA, especially when such interactions are established within the rRNA of the small ribosomal subunit in the unprocessed regions in the intermediates (e. g. 17S rRNA) that produce mature 16S rRNA (Shetty *et al.*, 2014) would be of interest. Furthermore, the study of mechanistic aspects of how 3GC pairs are involved in IF3 release would be of much importance.

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