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MINIREVIEW

Initiator Transfer RNAs

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INTRODUCTION

How does the cellular protein synthetic machinery distinguish initiator tRNA from all of the other tRNAs? What is the mechanism by which the initiator tRNA is sequestered for use exclusively in initiation of protein synthesis? Much interest has centered on questions of the sequence and/or structural information in the tRNA that is necessary for specifying the many distinctive properties of initiator tRNAs and the molecular basis by which the various proteins and/or components of the protein synthetic machinery utilize this information to distinguish initiator tRNAs from other tRNAs. Recent work, involving site-specific mutagenesis and analysis of the properties of the mutant tRNAs in vitro and in vivo, has led to identification of the sequence and structural features important in a eubacterial initiator tRNA. Here, I summarize this work and some of the implications of the results. Progress is also being made on initiator tRNAs from eukaryotic cytoplasm. This is included in another review (24).

SPECIAL PROPERTIES OF INITIATOR tRNAs

Initiation of protein synthesis occurs universally with the amino acid methionine or its derivative formylmethionine. Of the two classes of methionine tRNAs present in all organisms, the initiator is used exclusively for initiation of protein synthesis, whereas the elongator is used for insertion of methionine into internal peptidic linkages (7, 8, 15). In eubacteria and in eukaryotic organelles such as chloroplasts and mitochondria, the initiator tRNAs are used as formylmethionyl-tRNA (fMet-tRNA). In the cytoplasmic protein synthesis system of eukaryotes and in archaebacteria, they are used as methionyl-tRNA (Met-tRNA) without formylation. Although protein synthesis is initiated with either formylmethionine or methionine, very few proteins contain formylmethionine or methionine at their N termini. In most cases, the formyl group and the methionine residue are removed even as the nascent polypeptide chain is being synthesized on the ribosome (15).

To fulfill their special function, initiator tRNAs possess a number of highly specific properties which are different from those of elongator tRNAs (Fig. 1). For eubacterial initiator tRNAs, these properties include the following.

(i) Eubacterial initiator Met-tRNAs are formylated to fMet-tRNA by methionyl-tRNA transformylase (3) (step 2 of Fig. 1). This enzyme is specific for the initiator tRNA species.

(ii) Initiator tRNAs bind directly to the P site on the ribosome (step 3 of Fig. 1). In contrast, elongator tRNAs bind first to the A site and are translocated to the P site. Binding of initiator tRNAs to the P site requires the involvement of the initiation factors IF2 and IF3 (8). Whether IF2 distinguishes initiator tRNA from other tRNAs solely on the basis of the formylmethionine moiety and whether specific sequences in the tRNA are also involved is not known (32, 39). IF3 is thought to interact primarily with the anticodon stem and loop of the initiator tRNA (12). The genes for IF2 and IF3 have been cloned, and that for IF2 has been shown to be essential for growth of *Escherichia coli* (16).

(iii) Initiator tRNAs are sequestered for use in initiation and are excluded from binding to the A site on the ribosome. Binding to the ribosomal A site requires first the formation of a ternary complex comprising the elongation factor EF-Tu, GTP, and aminoacyl-tRNAs. The *E. coli* initiator Met-tRNA binds very poorly to EF-Tu · GTP (23) (step 4 of Fig. 1). In the formylated form (fMet-tRNA), affinity for EF-Tu is reduced even further.

(iv) Eubacterial initiator fMet-tRNAs are uniquely resistant to hydrolysis by peptidyl-tRNA hydrolase (14) (step 5 of Fig. 1). By hydrolyzing the ester linkage in peptidyl-tRNAs that have dissociated from the ribosome, this enzyme allows the tRNAs to recycle instead of accumulating as peptidyl-tRNAs and, therefore, plays an important role in protein synthesis (1). The resistance of eubacterial fMet-tRNA to this enzyme is a necessary property, given the utilization of fMet-tRNA for initiation.

STRUCTURE OF INITIATOR tRNA

Along with the special properties described above, eubacterial initiator tRNAs also possess unique sequence and/or structural features (25) that are not found in elongator tRNAs (Fig. 2). These include (i) the absence of a Watson-Crick base pair between nucleotides 1 and 72 at the end of the acceptor stem; (ii) the presence of a sequence of three guanines and three cytosines at the bottom of the anticodon stem, forming three consecutive G:C base pairs; and (iii) the presence of a purine 11:pyrimidine 24 base pair in the dihydrouridine stem, in contrast to a pyrimidine 11:purine 24 base pair in other tRNAs. At least two and most often all three of these features are found in chloroplast and mitochondrial initiator tRNAs, which also initiate protein synthesis with fMet-tRNA.

The crystal structure of the major *E. coli* initiator tRNA species (5), $tRNA_1^{fMet}$, has been determined to a resolution of 3.5 Å (0.35 nm) (40). The overall three-dimensional folding pattern of $tRNA_1^{fMet}$ is similar to that of yeast $tRNA^{Phe}$; however, the *E. coli* initiator tRNA structure differs from the yeast tRNA in three regions: (i) the CCA end, (ii) the dihydrouridine loop, and (iii) the anticodon loop. The unusual resistance of *E. coli* initiator tRNA to cleavage by S1 nuclease, compared with that of elongator tRNAs, also suggests a special structure for the anticodon loop of the initiator tRNA (41).

RELATIONSHIP BETWEEN STRUCTURE AND PROPERTIES OF INITIATOR tRNAs

Earlier studies on *E. coli* initiator tRNA, using chemical modification of C to U with bisulfite or anticodon replacement with RNA ligase, provided valuable information on the crucial

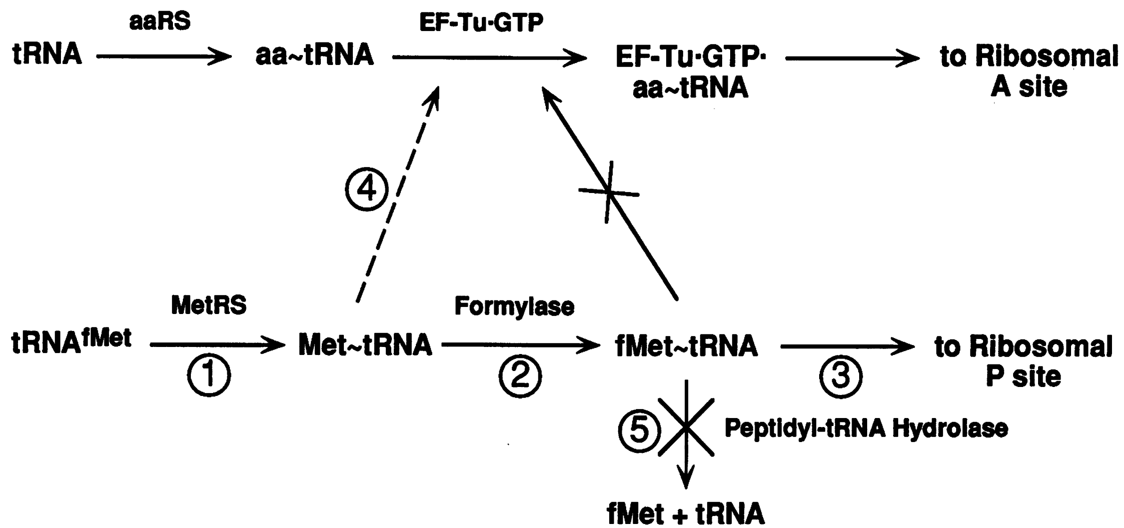


FIG. 1. Different properties of elongator (top) and initiator (bottom) tRNAs in eubacteria.

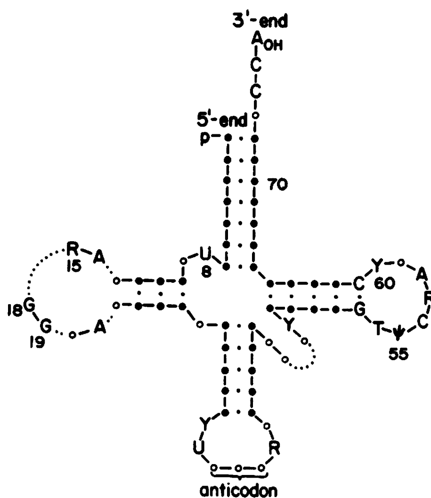
role of the anticodon sequence in aminoacylation by methionyl-tRNA synthetase (26). Since bisulfite reacts only with C residues that are unpaired and unstacked, identification of reactive C residues also provided structural information and some information on the possible role of the C1xA72 mismatch at the end of the acceptor stem (27) on initiator tRNA function.

More recent information has come from site-specific mutagenesis of the initiator $tRNA_2^{fMet}$ gene (13) followed by analysis of the properties of the mutant tRNAs in vitro and in vivo. $tRNA_2^{fMet}$ differs from $tRNA_1^{fMet}$ only in having adenosine instead of 7-methyl guanosine at position 46. Several techniques developed recently and listed below have greatly facilitated the detailed in vitro and in vivo analyses of the mutant tRNAs.

(i) Mutant tRNAs can be overproduced in *E. coli* and purified readily from other tRNAs, including $tRNA_1^{fMet}$, by using a single step of polyacrylamide gel electrophoresis (30). Furthermore, the finding that *E. coli* B lacks the $tRNA_2^{fMet}$ species has allowed expression of mutant $tRNA_2^{fMet}$ genes in *E. coli* B and isolation of mutant tRNAs without contamination by wild-type $tRNA_2^{fMet}$ (21). This has greatly simplified the analysis of data for kinetic parameters in aminoacylation and formylation of the mutant tRNAs (17).

(ii) The effect of mutations on aminoacylation and formylation (33) of mutant tRNAs in vivo can be assessed directly (Fig. 3). This depends upon the finding that the three different forms of initiator tRNA (uncharged tRNA, aminoacyl-tRNA, and formylaminoacyl-tRNA) can be separated from each other by gel electrophoresis and identified by hybridization (33).

ELONGATOR tRNAs :



EUBACTERIAL INITIATOR tRNAs :

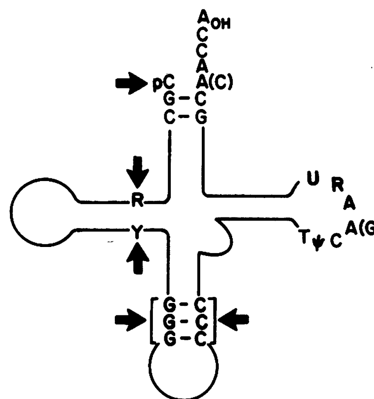


FIG. 2. Schematic diagram highlighting features unique to eubacterial initiator tRNAs. Left, cloverleaf structure of elongator tRNAs, indicating nucleotides common to all elongator tRNAs (R, purine; Y, pyrimidine). The small solid circles indicate the nucleotides involved in the formation of base pairs in the cloverleaf structure. Right, unique features in initiator tRNA indicated by arrows.

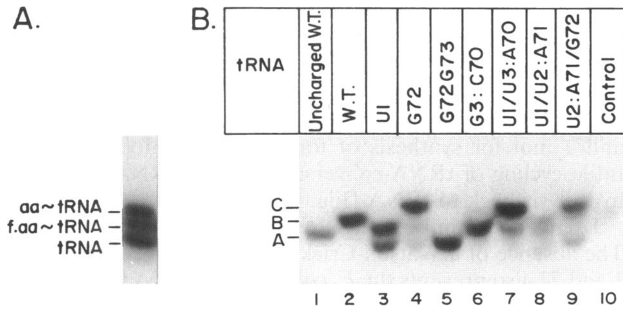


FIG. 3. Analysis of the effect of mutations on aminoacylation and formylation of the mutant tRNAs in vivo. (A) Separation of the three forms of initiator tRNA: tRNA, aminoacyl-tRNA (aa~tRNA), and formylaminoacyl-tRNA (f.aa~tRNA) by polyacrylamide gel electrophoresis under acidic conditions followed by detection of tRNA by RNA blot analysis using a labeled DNA probe. (B) RNA blot analysis of $tRNA_2^{fMet}$ from transformants carrying the wild-type gene or various mutant tRNA genes. Control, tRNA isolated from transformants carrying the plasmid vector without any tRNA gene.

(iii) The effect of mutations on function of mutant tRNAs in initiation and in the elongation steps of protein synthesis in vivo can also be analyzed. By using a chloramphenicol acetyl transferase gene in which the AUG initiation codon was changed to UAG as the reporter, a mutant initiator tRNA with a CAU→CUA anticodon sequence change was shown to initiate protein synthesis from UAG (36). Therefore, by coupling the CAU→CUA change in the anticodon with mutations in the main body of the tRNA, it is possible to determine the effect of the latter mutations on the overall activity of the mutant tRNAs in initiation in vivo (33, 35). Similarly, by expressing the same mutant tRNA genes in an *E. coli* strain carrying an amber mutation in the chromosomal *lacZ* gene, it is possible to identify those mutations in the initiator tRNA that allow it to now bind to the ribosomal A site and act as an elongator (29, 33, 35).

(iv) The effect of mutations on the properties of mutant tRNAs in vivo can be studied in different *E. coli* strains. By examining these properties in a strain with a temperature-sensitive mutation in the gene for peptidyl-tRNA hydrolase (1) and comparing them with those of a parent strain, it is possible to identify mutations in the initiator tRNA which make it a substrate for peptidyl-tRNA hydrolase (17, 18, 33).

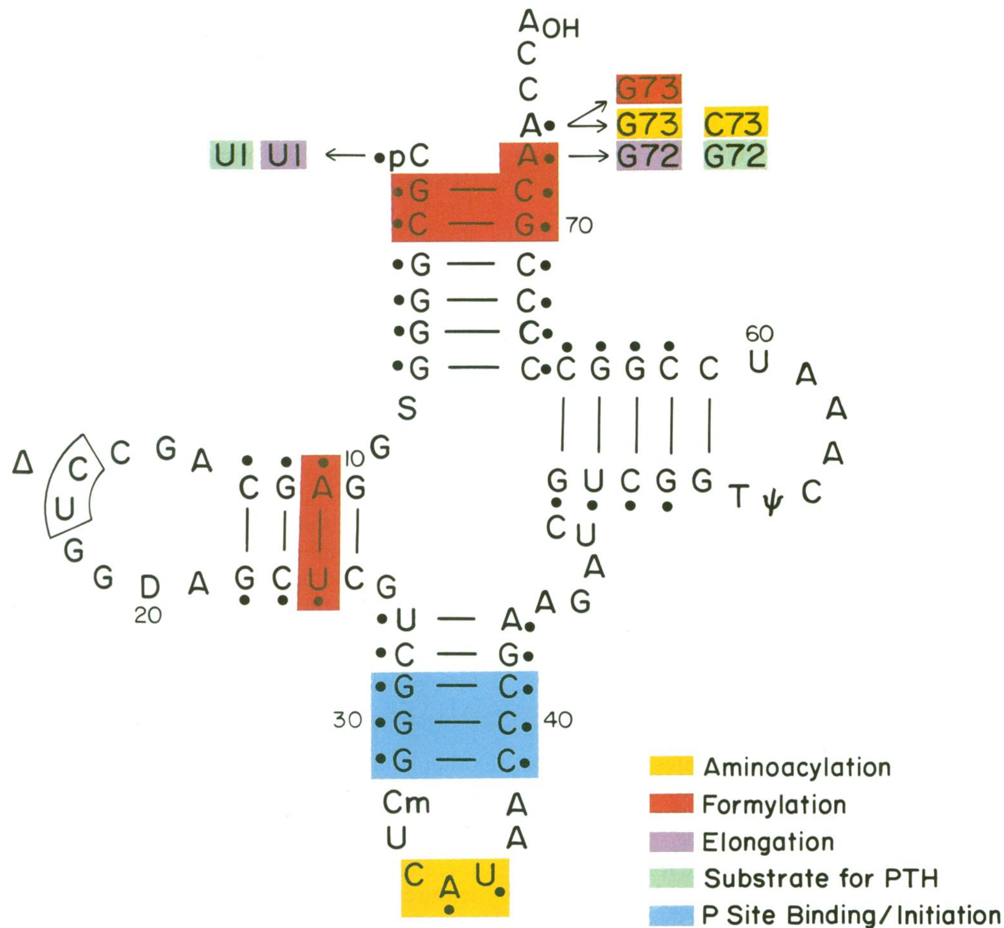


FIG. 4. Regions of *E. coli* $tRNA_2^{fMet}$ important for specifying the various special properties of the tRNA highlighted in color. Sites in the tRNA that have been mutagenized are indicated by dots (substitution) or a triangle (deletion). Arrows identify specific mutations which significantly affect the phenotype of the tRNA with respect to a particular function. As mentioned in the text, mutation of the A11:U24 base pair affects formylation kinetics. The exact role of this base pair is, however, not established and could be subtle.

The combined results of such in vitro and in vivo studies on a number of mutant tRNAs have allowed direct correlations between the phenotypic effect of a mutation in vivo with a specific step(s) along the pathway at which the mutant tRNA is defective. Figure 4 highlights sequence and/or structural elements in the *E. coli* initiator tRNA identified as being important for specifying each of its distinctive properties. Strikingly, these elements are clustered in two regions of the tRNAs, the acceptor stem and the anticodon stem and loop.

Recognition of Met-tRNA by Met-tRNA transformylase. The sequence and/or structural elements important for formylation by Met-tRNA transformylase are mostly clustered at the end of the acceptor stem (11, 17, 20). The key determinants appear to be a mismatch (as found in the wild-type tRNA) or a weak base pair between nucleotides 1 and 72, a G:C base pair between nucleotides 2 and 71, and a C:G (or, less preferably, a G:C) base pair between nucleotides 3 and 70. Mutations in the G4:C69 base pair also affect formylation kinetics, but they have less of an effect than mutations at the above positions. Mutations at position 73 affect formylation minimally, except for the G73 mutation (11, 17, 18). Because mutations of A73 to C or U have little or no effect, the effect, specifically, of G73 is likely due to negative interactions and/or an altered structure (18).

The nature of the mismatch between nucleotides 1 and 72 is less important for formylation than the fact that they cannot form a strong Watson-Crick base pair. tRNAs carrying the wild-type CxA mismatch or virtually any mismatch are good substrates, whereas tRNAs carrying strong base pairs, such as C1:G72 or G1:C72, are extremely poor substrates. These and other results suggest that nucleotides 1 and 72 must be unpaired for formylation to occur (11, 17, 18).

Outside of the acceptor stem, the only mutant with an alteration in kinetic parameters for formylation (K_m up by a factor of about 7) is the A11:U24-to-C11:G24 mutant (20).

Utilization of fMet-tRNA in initiation. The three consecutive G:C base pairs in the anticodon stem are important for targeting the initiator fMet-tRNA to the P site on the ribosome. Mutations here do not affect aminoacylation, formylation, or binding of fMet-tRNA to the initiation factor IF2. The mutants are defective specifically at the step of binding to the ribosomal P site (30). The activity of mutant tRNAs in vivo parallel those for ribosome binding in vitro. Simple transversion of the G30:C40 base pair to C30:G40 in a mutant tRNA also carrying the CAU→CUA anticodon sequence change lowers activity in initiation by approximately 25-fold without affecting aminoacylation or formylation of the tRNA (22). It is possible that the three consecutive G:C base pairs in the anticodon stem are signals for the initiation factor IF3 to select the initiator tRNA from the other tRNAs at the P site (12).

Protection of fMet-tRNA from hydrolysis by PTH. Besides its importance in formylation of the tRNA, the absence of a base pair between nucleotides 1 and 72 also protects fMet-tRNA from the hydrolytic action of peptidyl-tRNA hydrolase (PTH). Mutants of the initiator tRNA with U:A, A:U, C:G, or G:C base pairs at this position are all substrates for PTH in vivo and in vitro (6, 17, 18, 27). For the mutant with a U1:A72 base pair, a striking consequence is a significant accumulation of uncharged tRNA in vivo (Fig. 3B, lane 4) (17). This is because the mutant tRNA, still a good substrate for Met-tRNA transformylase, is converted to fMet-tRNA, which can now be hydrolyzed by PTH to formylmethionine and tRNA (Fig. 1). In an *E. coli* strain with a temperature-sensitive mutation in PTH (1), there is no accumulation of uncharged mutant tRNA (17).

Thus, another important role of the C1xA72 mismatch in eubacterial initiator tRNA is to help maintain high steady-state levels of fMet-tRNA in vivo by preventing its hydrolysis by PTH. A high cellular level of fMet-tRNA may be necessary for efficient translation initiation, a step thought to be rate limiting in *E. coli* (8). The resistance of *E. coli* fMet-tRNA towards PTH would also prevent waste of energy (2 mol of ATP used per mol of fMet-tRNA synthesized [1 mol for aminoacylation and 1 mol for synthesis of formyltetrahydrofolate]) through futile cycling of tRNA conversion to fMet-tRNA followed by hydrolysis back to tRNA (Fig. 1).

Exclusion of initiator tRNA from acting as an elongator. The absence of a Watson-Crick base pair between nucleotides 1 and 72 also prevents the *E. coli* initiator tRNA from acting as an elongator and thereby sequesters it for use exclusively in initiation (31). Single mutants that have either a U1:A72 or a C1:G72 base pair act as elongators in vitro, whereas a double mutant with a U1xG72 mismatch does not. The G72 mutant with a strong C1:G72 base pair is more active as an elongator in vitro than the U1 mutant with a weak U1:A72 base pair. This is also reflected in the relative affinities of the Met-tRNAs derived from these two mutants for EF-Tu · GTP (G72 > U1 > U1G2 = wild-type tRNA). Thus, the U1 and G72 mutant tRNAs are active in elongation because they bind EF-Tu · GTP, in contrast to the wild-type tRNA, which binds EF-Tu · GTP very weakly, if at all.

By coupling mutations in the main body of the tRNA with a CAU→CUA anticodon sequence change, a number of mutant tRNAs have been analyzed for their ability to act as amber suppressors and, therefore, as elongators in vivo. These studies confirm the results of in vitro analyses and show that all the mutants that have a 1:72 base pair are active in elongation in vivo in *E. coli* and in *Saccharomyces cerevisiae* (10, 19, 29, 35).

Role of the unique features of *E. coli* initiator tRNA in its function. Of the three features unique to eubacterial initiator tRNAs (Fig. 2), the roles of two of them are established. (i) The GGG:CCC sequence in the anticodon stem is important specifically for targeting the tRNA to the P site on the ribosome (30). (ii) Remarkably, the lack of a Watson-Crick base pair between nucleotides 1 and 72 is important for specifying the other three of its four distinctive properties (Fig. 4). Thus, three separate *E. coli* proteins (Met-tRNA transformylase, peptidyl-tRNA hydrolase, and EF-Tu) utilize the same structural feature, unique to eubacterial, chloroplast, and most mitochondrial initiator tRNAs, to distinguish the initiator tRNA from other tRNAs. This explains how a relatively limited number of nucleotides in the tRNA can specify all of the properties needed for initiation of protein synthesis. Further confirmation of this comes from the finding that introduction of the minimal sequences necessary for formylation and for P site binding onto elongator methionine and glutamine tRNAs allows these mutant tRNAs to act as initiator tRNAs in *E. coli* (10, 34).

The role of the third feature, the purine 11:pyrimidine 24 base pair in the D stem, is not as well established. Mutation of A11:U24 to C11:G24 results in an approximately sevenfold increase in K_m in the formylation reaction (20). However, other studies suggest that the importance of the A11:U24 base pair for formylation depends upon the sequence context in and around the D stem. The role of the A11:U24 base pair in eubacterial initiator tRNA structure and/or function could, therefore, be more subtle than those of the other two unique features.

CRITICAL ROLE OF FORMYLATION OF INITIATOR tRNA

Besides the anticodon stem mutants, the other mutations that affect initiation significantly lie in the acceptor stem. Here,

the effect of mutation depends upon the site of mutation and the nature of the mutation. There is an excellent correlation between formylation of tRNAs and their activity in initiation. Mutations at the first, second, and third base pairs in the acceptor stem which affect formylation greatly lower the activities of mutant tRNAs in initiation in vivo (35). Thus, formylation of initiator tRNA is important for initiation of protein synthesis in *E. coli*. The findings that disruption of the gene for Met-tRNA transformylase results in *E. coli* cells that grow extremely slowly at 37°C (an approximately eight- fold increase in doubling time in rich medium) and fail to grow at 42°C are consistent with this conclusion (9).

Does formylation of initiator tRNA in *E. coli* simply provide a positive determinant allowing the initiation factor IF2 to distinguish it from other tRNAs, or does formylation also contribute towards sequestration of the tRNA for initiation by blocking its binding to EF-Tu · GTP? In other words, is formylation of the initiator tRNA also necessary to completely block it from acting as an elongator in vivo? Some evidence for this comes from analysis of *E. coli* initiator tRNA mutants carrying the CAU→CUA anticodon sequence change. This mutant initiator tRNA, which has a C1xA72 mismatch at the end of the acceptor stem, does not usually suppress amber codons in vivo (35). However, if the Met-tRNA transformylase activity in cells is reduced drastically, the mutant tRNA now suppresses amber codons to some extent, presumably because most of the initiator tRNA in the cell is now present as aminoacyl-tRNA (10). Results of in vitro studies suggest, however, that simply blocking formylation of wild-type initiator tRNA may not be enough to allow it to act as an elongator. When wild-type initiator tRNA is added to an in vitro protein synthesis system as methionyl-tRNA, there is little transfer of methionine into internal peptidic linkages (31). It is possible that the in vitro studies are not sensitive enough to detect very low levels of activity. Further studies are necessary to evaluate the contribution of formylation to sequestration of the tRNA for initiation.

MUTANTS OF INITIATOR tRNA THAT ARE ACTIVE BOTH IN INITIATION AND IN ELONGATION

Although the *E. coli* initiator tRNA has been sequestered for use exclusively in initiation of protein synthesis, some of the mutant tRNAs, for example the U1 mutant with a U1:A72 base pair, can act both as initiators and as elongators (35). Thus, while there may be selective advantages to a cell in sequestering a tRNA for use in initiation, activities of a tRNA in initiation and in elongation are not by themselves mutually exclusive. This may explain why some of the mitochondrial DNAs, which have strong constraints on genome size and, thereby, on the number of tRNA genes, encode only one methionine tRNA. In such mitochondrial systems, the same methionine tRNA may act both as initiator (in the formylated form) and as elongator (in the unformylated form).

METHIONINE AS THE UNIVERSAL INITIATING AMINO ACID

Why is methionine the amino acid universally used for initiation of protein synthesis? Mutants of *E. coli* initiator tRNA which have changes in the anticodon sequence and which are, therefore, aminoacylated with amino acids other than methionine (28) can initiate protein synthesis with glutamine, isoleucine, phenylalanine, valine, etc. (2, 36). Thus, there is, a priori, no reason for restricting the initiating amino acid to methionine or the initiator codon to AUG. However, the effect of overproduction of methionyl-tRNA synthetase on activity of the CAU→CUA anticodon sequence mutant of *E. coli* initiator tRNA in vivo shows that initiation with methionine is much more efficient than initiation with glutamine (37). Other studies in *E. coli* indicate that initiation with methionine is better than that with isoleucine, phenylalanine, or valine (2). Thus, methionine may well be the best amino acid for initiation of protein synthesis in *E. coli*.

In eukaryotic systems, yeast initiator tRNA aminoacylated with methionine initiates protein synthesis in vitro, whereas the same tRNA aminoacylated with isoleucine does not (38). In mammalian cells, a CAU→CUA anticodon sequence mutant of human initiator tRNA, which is aminoacylated with glutamine, does not initiate protein synthesis in vivo (4). This is unlike the situation in *E. coli* (36). While any generalization based on two examples must remain tentative, it is possible that the protein-synthesizing system in eukaryotic cytoplasm has evolved in such a way as to initiate protein synthesis only with methionine.

PERSPECTIVES

The key features in a eubacterial initiator tRNA necessary for specifying its many distinct properties have been identified. This has provided a rather simple answer to the question "How can a tRNA have the same 'overall' secondary and tertiary folding as other tRNAs and yet have so many properties that are distinct?" Three separate *E. coli* proteins—methionyl-tRNA transformylase, peptidyl-tRNA hydrolase, and elongation factor EF-Tu—use the same structural feature unique to eubacterial, chloroplast, and most mitochondrial initiator tRNAs to distinguish the initiator tRNA from all other tRNAs. It should be interesting to determine the molecular mechanisms underlying these discriminations and discrimination by the initiation factors IF2 and IF3 and by the ribosome.

A number of mutant initiator tRNAs defective at specific steps along the initiation pathway have been characterized. Identification and analysis of suppressors which can rescue the defect in these tRNAs should provide much useful information on the translational machinery and could also lead to identification of new components of the machinery.

The mutant tRNAs used in this and other work initiate protein synthesis with codons other than AUG and amino acids other than methionine. *E. coli* cells carrying these tRNAs should be useful for the large-scale production of eukaryotic proteins that have specific amino acids at their N termini (2, 36). Furthermore, mutant initiator tRNAs which can be overproduced may have the potential of subverting the cellular machinery in such a way as to facilitate the translation of a desired mRNA (37).

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