From elongator tRNA to initiator tRNA
(tRNA identity switch/methionine tRNA/glutamine tRNA/formylation/ribosomal P site)

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ABSTRACT We show that the two most important properties needed for a tRNA to function in initiation in Escherichia coli are its ability to be formylated and its ability to bind to the ribosomal P site. This conclusion is based on conversion of two different elongator tRNAs to ones that can act as initiators in E. coli. We transplanted the features unique to E. coli and eubacterial initiator tRNAs to E. coli elongator methionine tRNA (tRNA\textsuperscript{Met}) along with an anticondor sequence change and analyzed their activities in initiation in E. coli. Introduction of a C1-A72 mismatch at the end of the acceptor stem of tRNA\textsuperscript{Met}, which generates the minimal features necessary for formylation, produces a tRNA with very low activity in initiation. Subsequent introduction of three consecutive G-C base pairs at the bottom of the anticondor stem, which is necessary for ribosomal P site binding, produces a tRNA with significant activity in initiation. Furthermore, introduction of the features necessary for formylation and for ribosomal P site binding into E. coli elongator glutamine tRNA produces a tRNA that initiates protein synthesis in E. coli.

Of the two classes of methionine tRNAs present in all organisms, the initiator is used for initiation of protein synthesis, whereas the elongator is used for inserting methionine into internal peptide linkages (1). Because of their unique function, initiator tRNAs possess several properties distinct from those of elongator tRNAs. In eubacteria and in eukaryotic organelles such as mitochondria and chloroplasts, the initiator is used as formylmethionyl-tRNA (fMet-tRNA). Following aminoclylation of the initiator tRNA (tRNA\textsuperscript{Met}), the methionyl-tRNA (Met-tRNA) is specifically formylated to fMet-tRNA by methionyl-tRNA transformylase. The fMet-tRNA binds to and elongator tRNAs, which first bind to the A site (2, 3). In addition, initiator tRNAs do not bind to the A site on the ribosome and therefore cannot act at the elongation step of protein synthesis.

Along with these special properties, eubacterial initiator tRNAs also possess unique sequence and/structural features that are not found in elongator tRNAs (4). 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 anticondor 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-pyrimidine-24 base pair in other tRNAs.

Functional studies on mutants of Escherichia coli initiator tRNA have provided information on features important for specifying their distinctive properties (5, 6), including the sequence and/or structural features that are crucial for formylation and for binding of the tRNA to the P site on the ribosome. The key features necessary for formylation appear to be a mismatch or a weak base pair between nucleotides 1 and 72, a G2-C71 base pair, and a C3-G70 base pair (7, 8). Mutations in G4-C69 and A11-U24 base pairs affect formylation kinetics but have less of an effect than mutations at the above positions (7). Once the tRNA is formylated, the feature important for targeting the tRNA to the P site on the ribosome appears to be the three consecutive G-C base pairs in the anticodon stem (9).

Given the many distinctive properties of initiator tRNAs, a question of interest is: what are the minimal alterations in an elongator tRNA that are needed to convert it into an initiator tRNA? In this paper we show that introduction of the features necessary for formylation of the tRNA and for targeting the tRNA to the P site on the ribosome allows the E. coli elongator methionine tRNA (tRNA\textsuperscript{Met}) and glutamine tRNA (tRNA\textsuperscript{Gln}) to initiate protein synthesis in E. coli.

MATERIALS AND METHODS

Mutagenesis of Elongator Methionine and Glutamine tRNA Genes. Mutant tRNA genes were generated by using oligonucleotide-directed mutagenesis (6, 9). For mutant elongator methionine tRNA genes, a 93-nucleotide-long mutagenic primer was used to replace the (C27A28T29C30A31) (T39G40A41T42G43) initiating tRNA gene present in phase M13 DNA (7) with the C1-A72 mutant of E. coli elongator methionine tRNA gene. The resulting mutant elongator methionine tRNA gene was then used to generate the other elongator mutant tRNA genes listed in Table 1. For mutant glutamine tRNA genes, a 93-nucleotide-long mutagenic primer was used to replace the T35A36 mutant initiating tRNA gene present in M13 DNA. The mutant tRNA genes were characterized by sequencing of the entire tRNA gene. All of the mutant tRNA genes have the same promoter and terminator sequences as in the initiator tRNA gene.

Vectors and Strains Used. The mutant tRNA genes used were cloned into either pRSV CATam1.25 (a pBR322-based vector) or pTZ19R (a pUC-based vector). The E. coli genes encoding glutaminyl- and methionyl-tRNA synthetases were cloned into pAC1 (a pACYC-based vector) (10). The E. coli strains used were either CA274 (9) or TG1.

Assays for Initiation Activity of Mutant Elongator Methionine and Glutamine tRNAs. The mutant tRNAs studied here all have a CAU \rightarrow CUA anticondor sequence change. This allows assay for their ability to initiate protein synthesis from UAG by using a mutant chloramphenicol acetyltransferase (CAT) gene (CATam1.2.5) in which the initiator AUG codon is changed to UAG (10, 11). Production of CAT protein was measured by assay for CAT activity in cell extracts and by immunoblot analysis (11).

Detection of Mutant tRNAs by Northern Blot Hybridization. Total tRNAs isolated from E. coli at pH 8.0 and at room temperature were fractionated by gel electrophoresis on a 12% nondenaturing polyacrylamide gel as described (7) prior to hybridization.

Abbreviation: CAT, chloramphenicol acetyltransferase.

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Table 1. List of E. coli elongator methionine tRNA mutants

<table>
<thead>
<tr>
<th>Mutant designation</th>
<th>Nature of mutations</th>
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<tbody>
<tr>
<td>M:i1</td>
<td>CltA72/T35A36</td>
</tr>
<tr>
<td>M:i2</td>
<td>CltA72/G29G30G31C39C40C41/T35A36</td>
</tr>
<tr>
<td>M:i3</td>
<td>CltA72/All1-T24/G29G30G31C39C40C41/T35A36</td>
</tr>
</tbody>
</table>

Separation of Uncharged, Aminocyl-, and Formylarninoacyl tRNAs. Total tRNAs isolated from E. coli under acidic conditions were subjected to polyacrylamide gel electrophoresis at pH 5.0 and at 4°C (12). The various forms of the tRNAs were detected by Northern blot hybridization by using sequence-specific deoxyribonucleotide probes labeled at the 5' end with 32P.

RESULTS

Mutants of E. coli Elongator Methionine tRNA. Three elongator tRNA mutants were used (Fig. 1 Left and Table 1). These mutants, designated M:i1, M:i2, and M:i3, contained respectively one, two, or all three of the features unique to E. coli initiator tRNA. M:i1 contained a CltA72 mismatch instead of a G1-C72 base pair. Because the second and third base pairs in the acceptor stem of E. coli RNA Met and tRNA Met are identical, the M:i1 mutation generates the minimal features necessary for recognition of the tRNA by the formylating enzyme. M:i2 contained additional changes in the anticodon stem such that it has three G-C base pairs at the bottom of the anticodon stem. M:i3 further contained an A11-U24 base pair in the dihydrouridine stem instead of a C11-G24 base pair.

For functional studies in initiation from UAG codon in vivo, the above mutant tRNAs also contained an anticodon sequence mutatation from CAU → CUA (11). Because of the anticodon sequence change, all three of the elongator tRNA mutants studied are very poor substrates for E. coli methionyl-tRNA synthetase (6, 13, 14). Although the elongator tRNA mutant with the anticodon change alone is aminocylated in E. coli with lysine (15), the effect on aminocylation with lysine of the CltA72 mismatch common to the three mutants studied here is unknown (16). Since the E. coli initiator tRNA mutant with the same anticodon sequence change is aminocylated with glutamine in vitro and in vivo (6, 12, 13) and the presence of a CltA72 mismatch favors aminocylation by E. coli glutaminyl-tRNA synthetase (6, 17, 18), it is likely that these mutant tRNAs are aminocylated at least to some extent with glutamine. Also, we had found that some mutants of E. coli initiator tRNA that are defective in formylation and inactive in initiation when aminocylated with glutamine become partially active in cells overproducing methionyl-tRNA synthetase (19). Therefore, we studied the effect of overproduction of either enzyme on activity of the mutant elongator tRNAs in initiation in E. coli.

Activity of the Mutant Elongator Methionine tRNAs in Initiation in E. coli. Extracts from cells containing the CATam1.2.5 reporter gene and one of the mutant tRNA genes were assayed for CAT activity and CAT protein levels as a measure of the activity of the mutant tRNAs in initiation. Table 2 shows that none of the three elongator tRNA mutants are active in initiation in cells that are not overproducing any of the aminocyl-tRNA synthetases (column −) or in cells that are overproducing glutaminyl-tRNA synthetase (+ GlnRS). In cells overproducing methionyl-tRNA synthetase (+ MetRS), however, the M:i1 mutant with the acceptor stem change has a low but detectable level of activity. In contrast, the M:i2 mutant with additional changes in the anticodon stem is quite active. The CAT activity in cell extracts is ~30% of that in cells transformed with a mutant initiator tRNA carrying the same anticodon sequence change. Interestingly, when the third unique feature of the initiator tRNA, an A11-U24 base pair, is introduced to generate the M:i3 mutant, no CAT activity is found in cell extracts. The reason for this is that these cells do not accumulate any M:i3 mutant tRNA.

Immunoblot analysis (20) of cell extracts for CAT protein (11) confirms the assays for CAT activity (Fig. 3). CAT protein is found in transformants that carry either the E. coli initiator tRNA mutant (FM/T35A36) or the elongator tRNA mutant M:i2. Synthesis of CAT protein with the M:i2 mutant requires overproduction of methionyl-tRNA synthetase.

Table 2. Relative CAT activities in extracts of transformed E. coli

<table>
<thead>
<tr>
<th>tRNA gene</th>
<th>Relative CAT activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
</tr>
<tr>
<td>FM/T35A36*</td>
<td>100</td>
</tr>
<tr>
<td>M:i1</td>
<td>nd</td>
</tr>
<tr>
<td>M:i2</td>
<td>0.8</td>
</tr>
<tr>
<td>M:i3</td>
<td>0.3</td>
</tr>
<tr>
<td>Q:i2</td>
<td>10</td>
</tr>
</tbody>
</table>

Relative CAT activities (%) in extracts of E. coli CA274 transformed with pRSV plasmids carrying the CATam1.2.5 gene and various mutant tRNA genes including those listed in Table 1. The transformants also contained another plasmid that did not (−) or did (+) contain the gene for E. coli glutaminyl-tRNA synthetase (GlnRS) or E. coli methionyl-tRNA synthetase (MetRS).

*E. coli initiator tRNA Met* gene carrying the T35A36 mutation. CAT activity in cells containing this tRNA gene and not overproducing any of the aminocyl-tRNA synthetases is fixed at 100%.

1nd, Not determined. However, since these cells do not grow on plates containing chloramphenicol after 16 hr, they contain much less than 4% CAT (11).
(compare third and fourth lanes from left). In accordance with the CAT activity assays, a very small amount of CAT protein is present in extracts of cells that carry the M:i2 mutant and are also overproducing methionyl-tRNA synthetase (Fig. 3, rightmost lane). The similar levels of β-lactamase in the cell extracts suggest that the transformants all contained the vector carrying the CATaml1.2.5 gene and the respective mutant tRNA genes (11).

Aminoacylation and Formylation of M:i2 Mutant tRNA in E. coli. Recently, we described a method (12) to separate uncharged, aminoacylated and formylaminoacylated forms of tRNA from each other and to detect these various forms by using Northern blot analysis of tRNA isolated under conditions where the linkage between the amino acid and the tRNA is preserved. Application of this method to the M:i2 mutant tRNA shows that ≈50% of the M:i2 mutant tRNA is aminoacylated in vivo but none of it is formylated (Fig. 4, lane 3). In cells overproducing glutaminyl-tRNA synthetase, somewhat more of the tRNA is aminoacylated, presumably at least partially with glutamine; however, there is still no formylation of the aminoacyl-tRNA (lane 4). In contrast, in cells overproducing methionyl-tRNA synthetase, a substantial fraction of the tRNA is now converted to the formylated form (lane 5). This is most likely because overproduction of methionyl-tRNA synthetase leads to aminoacylation of the M:i2 mutant tRNA with methionine in vivo (19, 21–23) and that this tRNA is a much better substrate for methionyl-tRNA transformylase than the tRNA aminoacylated with other amino acids. The formylation of M:i2 tRNA only in cells overproducing methionyl-tRNA synthetase would explain its activity in initiation in these cells.

An alternate possibility is that the E. coli elongator methionine tRNA mutant M:i2 is active in initiation in cells that are not overproducing any aminoacyl-tRNA synthetase but that the CAT protein that is made is unstable because it is initiated with lysine. This possibility can be ruled out. Proteins with lysine or arginine at the N terminus are thought to be unstable in E. coli (24) because they are substrates for the E. coli leucine/phenylalanine-tRNA protein transferase (L/F-transferase). This enzyme attaches leucine or phenylalanine to the N terminus of such proteins and thereby destabilizes the proteins in vivo. We transformed an E. coli strain that carries a disruption in the gene for the L/F-transferase enzyme with the plasmid carrying the pRSV CATaml1.2.5 gene and the M:i2 mutant tRNA gene. Transformants still do

![Figure 2](biochemistry_varshney_et_al_1993.png)

**Fig. 2.** RNA blot hybridization of total tRNA isolated from transformants carrying the M:i2 (lanes 1 and 3) or the M:i3 (lanes 2 and 4) mutant elongator methionine tRNA genes on a pBR-based vector (lanes 1 and 2) or a high-copy pUC-based vector (lanes 3 and 4). The probe used was complementary to nucleotides 25–45 of the M:i2 or the M:i3 mutant tRNA.

![Figure 3](biochemistry_varshney_et_al_1993.png)

**Fig. 3.** Immunoblot analysis with rabbit anti-CAT and anti-β-lactamase antibodies of crude extracts from E. coli CA274 transformants carrying the CATaml1.2.5 gene and either the T35A36 mutant initiator tRNA gene or the various mutant elongator tRNA genes as indicated. The transformants also contained another plasmid which did (+) or did not carry the gene for E. coli methionyl-tRNA synthetase.

![Figure 4](biochemistry_varshney_et_al_1993.png)

**Fig. 4.** RNA blot hybridization of total tRNA isolated from E. coli transformants carrying the M:i2 mutant elongator tRNA gene. The tRNAs, isolated under acidic conditions, were separated by electrophoresis on a 6.5% acid-urea polyacrylamide gel at 4°C (12). The probe used was complementary to nucleotides 21–48 of the M:i2 mutant tRNA. Lanes: 1 and 2, marker tRNAs isolated from TG1 cells by using a high-copy pUC vector (lane 1) and treated at alkaline pH to deacylate the charged tRNA prior to electrophoresis (lane 2); 3–5, tRNA isolated from CA274 transformants containing an additional plasmid that had no aminoacyl-tRNA synthetase gene (lane 3), the E. coli glutaminyl-tRNA synthetase gene (lane 4), or the E. coli methionyl-tRNA synthetase gene (lane 5).
of three consecutive G-C base pairs at the bottom of the anticodon stem generates features important for P site binding. This tRNA has significant activity in initiation. The amount of CAT activity in extracts of cells carrying the mutant elongator tRNA is ≈30% of that in cells carrying the *E. coli* initiator tRNA mutant with the corresponding anticodon sequence change.

The elongator methionine tRNA mutant M:i2 is active in initiation only in cells that are overproducing methionyl-tRNA synthetase. The reason for this is that the M:i2 mutant tRNA is formylated only when it is aminoacylated with methionine (Fig. 4, lane 5). Since the M:i2 mutant tRNA is a very poor substrate for methionyl-tRNA synthetase because of the anticodon sequence change (14), aminoacylation of this tRNA with methionine requires overproduction of the enzyme. These results are similar to those obtained with the T35A36/G72 mutant of *E. coli* initiator tRNA (19). The strong correlation between formylation of the M:i2 mutant tRNA (Fig. 4, lane 5) and activity in initiation (Table 2) supports our previous conclusion, based on analysis of mutants of *E. coli* initiator tRNA, that formylation of initiator tRNA is important for initiation of protein synthesis (11). Further support for this comes from the finding that the Q:i2 mutant derived from glutamine tRNA, which is formylated in *E. coli* under all conditions (Fig. 5), shows partial activity in initiation, even in cells not overproducing glutaminyl- or methionyl-tRNA synthetase. The work of Guillon *et al.* (25), showing that disruption of the gene for methionyl-tRNA transformylase in *E. coli* results in cells that grow extremely slowly, is also consistent with this conclusion.

In contrast to the methionine elongator tRNA mutant M:i2, which is formylated in vitro only when aminoacylated with methionine, the glutamine tRNA mutant Q:i2 is formylated even when aminoacylated with glutamine (Fig. 5). Thus, the Q:i2 mutant is a better substrate for the formylating enzyme than the M:i2 mutant. Both tRNAs contain the same nucleotides at positions 1, 72, and base pairs 2-71 and 3-70, which are considered important for formylation (7, 8, 26). The major difference is the absence in the M:i2 mutant and the presence in the Q:i2 mutant of base pairs G4-C69 and G5-C68 found in the *E. coli* initiator tRNA. Mutations of the G4-C69 and G5-C68 base pairs to C-G base pairs in an initiator tRNA sequence background have a relatively small effect (7), lowering the *V*<sub>max</sub>/*K*<sub>m</sub> parameters in formylation by approximate factors of 4.7 and 2.2, respectively. However, a similar mutation of the G4-C69 base pair in an elongator methionine tRNA background has a more severe effect, lowering *V*<sub>max</sub>/*K*<sub>m</sub> by about 2 orders of magnitude (8). Thus, the relative importance of the G4-C69 and possibly the G5-C68 base pairs in formylation depends on the tRNA context (8). The total lack of formylation of the M:i2 mutant tRNA in cells overproducing GltRS is, therefore, likely due to the combined effect of having glutamine instead of methionine and not having the G4-C69 and possibly the G5-C68 base pairs in the tRNA.

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