Mutants of *Escherichia coli* formylmethionine tRNA: A single base change enables initiator tRNA to act as an elongator *in vitro*

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**ABSTRACT** We show that the absence of a Watson–Crick base pair at the end of the amino acid acceptor stem, which is a hallmark of all prokaryotic initiator tRNAs, is one of the key features that prevents them from acting as an elongator in protein synthesis. We generated mutants of *Escherichia coli* formylmethionine tRNA that have a base pair at the end of the acceptor stem. The mutants generated were C1 → T1, which had a U–A base pair, A72 → G72, which had a C–G base pair, and the C1A72 → T1G72 double mutant, which lacked the base pair. After aminoacylation, the activity of these and other mutant initiator methionyl-tRNAs (Met-tRNAs) in elongation were assayed in a MS2 RNA-directed *E. coli* protein-synthesizing system and in binding to the elongation factor Tu (EF-Tu). Unlike wild-type initiator tRNA or the T1G72 double mutant, the T1 and G72 mutant Met-tRNAs were active in elongation, the G72 mutant being more active than the T1 mutant. The T1 and G72 mutant Met-tRNAs also formed a ternary complex with elongation factor EF-Tu-GTP, and their relative affinities for EF-Tu-GTP paralleled their activities in elongation. Combination of the T1 or G72 mutation with mutations in the GGG-CCC sequence conserved in the anticodon stem of initiator tRNAs led to a further increase in the activities of these mutant tRNAs in elongation such that one of these mutants was now almost as good an elongator as *E. coli* elongator methionine tRNA. Prokaryotic and eukaryotic initiator tRNAs contain a sequence of three guanines and three cytosines in the anticodon stem forming three consecutive G-C pairs. Prokaryotic initiator tRNAs share two additional unique features, the absence of a Watson–Crick base pair at the end of the acceptor stem and the presence of a purine 11:pyrimidine 24 base pair instead of a pyrimidine 11:purine 24 base pair in the dihydouridine stem (7). The strong conservation of these unique features suggests that these features account for one or more of the unique properties of prokaryotic initiator tRNAs.

We have used oligonucleotide-directed mutagenesis (8, 9) to remove two of the above features in *E. coli* tRNA^Met^ (10). Previously, we showed that the GGG-CCC sequence conserved in the anticodon stem of this tRNA was important for binding of the fMet-tRNA to the P site on the ribosome (11). In the current work, we generated several new mutant tRNAs and examined whether changes at either the end of the acceptor stem or in the conserved anticodon stem sequences allow these tRNAs to act as elongators. We show that single base mutants, T1 and G72, which contain a Watson–Crick base pair at the end of the acceptor stem, are active in elongation. These mutants bind the elongation factor Tu (EF-Tu) and their relative affinity (12) for EF-Tu-GTP parallels their activity in elongation. Changes in the GGG-CCC sequence conserved in the anticodon stem do not by themselves allow the initiator to act as an elongator. Thus, of the two unique features of *E. coli* initiator tRNA that have been altered, one is important for initiation (11), whereas the other is important in preventing the initiator tRNA from acting as an elongator.

**MATERIALS AND METHODS**

**Mutagenesis and Purification of Mutant tRNAs.** Mutagenesis of *E. coli* initiator tRNA^Met^ gene and purification of mutant tRNAs were as described (11).

**Aminoacylation and Isolation of Labeled Met-tRNA.** tRNAs were aminoacylated with either [35S]- or [3H]methionine using purified preparations of *E. coli* Met-tRNA synthetase. After incubation at 37°C for 10 min, 0.1 volume of 2.5 M NaOAc (pH 4.5) was added to the reaction mixture, the tRNA was extracted with phenol saturated with 10 mM NaOAc (pH 4.5) and ethanol precipitated, and aminoacyl-tRNA in the pellet was dissolved in 10 mM NaOAc (pH 4.5). The aminoacyl-tRNAs were dialyzed against 10 mM NaOAc (pH 4.5) for 16 hr at 4°C to remove any free radioactive methionine and stored at −70°C (13).

**Assay for Activity of Mutant tRNAs in Elongation.** MS2 RNA-directed protein synthesis was as described (14) with an S30 extract made from *E. coli* MRE 600 (15, 16). S30 extract was passed through Sephadex G-50, divided into aliquots, and stored in −70°C. MS2 RNA was prepared according to Goldman and Hatfield (14). The incubation mixture (37 μL)

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contained 1.5 A260 units of S30 extract, 50 mM ammonium acetate, (pH 7.0), 90 mM NH4Cl, 6 mM MgCl2, 8 mM 2-mercaptoethanol, 2 mM ATP, 0.3 mM GTP, 3.5 mM sodium phosphate buffer, 0.1 mM of each 20 amino acids including methionine, 0.8 A260 unit of crude E. coli tRNA, 37 mM ammonium acetate, 25–30 pmol of wild-type or mutant [35S]Met-tRNAs (2.5–5.5 × 10⁶ cpm/pmol), and 180–200 pmol of nonradioactive fMet-tRNAMet. Aliquots (6 µl) were removed for measuring alkali-stable trichloroacetic acid-precipitable radioactivity on a glass fiber filter.

**Tryptic Peptide Mapping of Proteins Synthesized in Vitro.** [35S]Methionine-labeled proteins made in vitro were recovered by alkali treatment of the incubation mixture followed by trichloroacetic acid precipitation. Protein was digested with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (14). The amount of radioactivity in tryptic digests was 4400 cpm for E. coli tRNA^Met^, 3600 cpm for the T29C30A31T39G40A41G72 mutant, and 2800 cpm for the G72 mutant, respectively. Half of each sample was used for electrophoresis. After electrophoresis (2 hr at 100 V with 120 mA), the paper was dried, cut into 1.27-cm strips, and assayed for radioactivity.

**Competition Assay for EF-Tu-GTP Binding.** E. coli EF-Tu-GDP was kindly provided by F. Jurnak (University of California, Riverside). Competition assay for EF-Tu-GTP binding (18) based on ribonuclease protection was carried out according to Louie et al. (12). EF-Tu-GTP was prepared from EF-Tu-GDP by exchange with GTP as described (12). Binding of [3H]- or [35S]met-tRNA to EF-Tu-GTP was carried out in a mixture (40 µl) containing 50 mM Tris (pH 7.4), 10 mM MgCl2, 150 mM NH4Cl, 5 mM dithiothreitol, 0.02% sodium azide, 3 mM phosphoenolpyruvate, 20 mM ATP, 10 µM GTP, 2 pmol of EF-Tu-GTP, and 7-35 pmol each of the competing [3H]- and [35S]met-tRNAs. After 15 min on ice to allow complex formation to reach equilibrium, the reaction mixture was treated with 4 µl of pancreatic RNase (1 mg/ml) for 30 sec. The RNase digestion was stopped by rapid addition of 10 µl of total E. coli RNA (1 mg/ml) and 1 µl of ice-chilled 10% trichloroacetic acid; the mixture was then left for 15 min of further incubation on ice. The precipitated RNAs were collected on a nitrocellulose filter (HA type, Millipore), washed thrice with chilled 10% trichloroacetic acid, and dried. The amount of radioactivity was measured simultaneously over three energy levels corresponding to [3H], [35S], and [3H] + [35S] speeas by using a Packard TRI-CAR 300C liquid scintillation counter. Spillover values were corrected for each isotope. Relative affinity of different mutant tRNAs toward EF-Tu-GTP represents the ratio of equilibrium dissociation constants between the mutant tRNAs (12, 18).

**RESULTS**

**Mutants of the tRNA^Met^ Gene.** The mutants used in this work—T1, G72, T1G72, T2A71, T1T2A71, T2A71G72, T29C30A31T39G40A41, T1T29C30A31T39G40A41, and T29C30A31T39G40A41G72—were obtained by using previously described procedures (11). These mutants contain changes at either the acceptor stem, the anticodon stem, or both (Fig. 1). The mutant tRNA genes were characterized by DNA sequencing of the entire tRNA gene and mutant tRNA genes were characterized by fingerprint analysis of [32P]labeled tRNAs (11).

**Function of Mutant tRNAs in Vitro.** The mutant tRNAs were purified by gel electrophoresis and aminoacylated with [35S]- or [3H]methionine before use for studies on their function (11).

**Activity as an elongator in protein synthesis.** Fig. 2 shows the time course of incorporation of [35S]methionine from the various mutant [35S]Met-tRNAs with changes at the end of the acceptor stem into protein.
Fig. 3 shows the effect of mutations in the GGG-CCC sequence conserved in the anticodon stem of all initiator tRNAs on activity of the mutant tRNAs in elongation. Mutations in the anticodon stem alone do not allow the mutant tRNA to act as an elongator (curve 2). However, coupling of the anticodon stem mutations with either the T1 or the G72 mutant increased the activity of both of the latter mutants in elongation (compare curve 4 with curve 3 and curve 6 with curve 5). The G72 mutant with changes in the anticodon stem sequence is now about 75% as active as elongator Met-tRNA<sub>Met</sub> (compare curve 6 and curve 7). As noted above, the T1 mutant is less active in elongation than the G72 mutant (Fig. 1). We have found that unlike the T1 mutant, the G72 mutant Met-tRNA<sub>Met</sub> is a poor substrate for E. coli Met-tRNA transformylase (the ratio of V<sub>max</sub>/K<sub>m</sub> for wild-type tRNA over the G72 mutant being about 500) (unpublished work). Therefore, one possible explanation for the lower activity of the T1 mutant compared to the G72 mutant in elongation is that most of the T1 mutant [35S]Met-tRNA<sub>Met</sub> added to the extract is converted to [35S]Met-tRNA, which cannot act in elongation. To rule out this possibility we combined the T1 mutation with the T2A71 mutation, which also makes the mutant Met-tRNA a very poor substrate for formylation (unpublished result; the ratio of V<sub>max</sub>/K<sub>m</sub> for T1 mutant over the T2T2A71 mutant being about 150) and asked whether the activity of the T1T2A71 mutant Met-tRNA<sub>Met</sub> in elongation is now the same as that of the G72 mutant. Results (Fig. 4) show that although introduction of the T2A71 mutation into either the T1 or the G72 mutant increases their activities in elongation, the activity of the T1T2A71 mutant in elongation is still substantially lower than that of the G72 mutant.

Fig. 4 also shows the results of an assay in which the same amount of radioactivity in the form of free [35S]methionine rather than [35S]Met-tRNA was added. The absence of any incorporation in this case shows that the observed incorporation of [35S]methionine from the Met-tRNAs is direct and not due to deacylation of the mutant Met-tRNAs to free [35S]methionine, which is then incorporated into protein.

FIG. 3. Time course of incorporation of [35S]methionine from the various mutant [35S]Met-tRNAs with changes at the end of the acceptor stem and/or in the anticodon stem into protein. Curve 1, MS2 RNA (control); curve 2, T29C30A31T39G40A41 mutant; curve 3, T1 mutant; curve 4, T1T29C30A31T39G40A41 mutant; curve 5, G72 mutant; curve 6, T29C30A31T39G40A41G72 mutant; curve 7, elongator tRNA<sub>Met</sub>.

FIG. 4. Time course of incorporation of [35S]methionine from the various mutant [35S]Met-tRNAs with changes at the end and the next base pair of the acceptor stem into protein. The control reaction contained the same amount of radioactivity in the form of free [35S]methionine instead of [35S]Met-tRNAs as in the other incubations.

Tryptic Peptide Mapping of Proteins Synthesized in Vitro. The predominant protein made in an MS2 RNA-directed E. coli protein-synthesizing system is the MS2 coat protein, which contains two internal AUG-encoded methionine residues (20). Therefore, tryptic mapping of [35S]methionine-labeled proteins made in vitro can be used to ask whether mutant E. coli initiator Met-tRNAs incorporate methionine only in response to AUG or to other codons such as GUG (3, 21). Two major peptides are present in trypsin digests of [35S]methionine-labeled proteins derived from either the G72 or the T29C30A31T39G40A41G72 mutant Met-tRNAs (Fig. 5). The electrophoretic mobility of these peptides and the relative ratio of radioactivity in these peptides are the same.
Table 1. Relative affinities of the various mutant Met-tRNAs for binding to EF-Tu-GTP

<table>
<thead>
<tr>
<th>tRNA</th>
<th>Affinity relative to wild-type tRNA&lt;sup&gt;Met&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>13.6</td>
</tr>
<tr>
<td>G72</td>
<td>155</td>
</tr>
<tr>
<td>T1G72</td>
<td>1.3</td>
</tr>
<tr>
<td>T29c30A1T39Q40A1G172tRNA&lt;sup&gt;Met&lt;/sup&gt; (elongator)</td>
<td>207</td>
</tr>
<tr>
<td>fMet-G72&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*The G72 mutant tRNA aminocacylated with [35S]methionine and subsequently formylated in the presence of a large excess of Met-tRNA transformylase.

+Under the conditions used for binding, the equilibrium dissociation constant of elongator tRNA<sup>Met</sup>-EF-Tu-GTP complex was 56 nM.

As those found in a corresponding digest of [35S]methionine-labeled protein derived from elongator Met-tRNA<sup>Met</sup>.

**Binding of Mutant Met-tRNAs to EF-Tu.** The binding affinities of the various mutant initiator tRNAs for EF-Tu-GTP were measured by using a competition assay (2) based on resistance of aminocyl-tRNAs complexed with EF-Tu-GTP toward ribonuclease (19). The relative affinities shown on Table 1 were calculated on the basis of competition between each mutant Met-tRNA and either wild-type initiator Met-tRNA, elongator Met-tRNA, or other mutant Met-tRNAs. The wild-type initiator tRNA and the T1G72 mutant, both of which lack the base pair at the end of the acceptor stem (Fig. 1), do not bind to EF-Tu-GTP, whereas the T1 and G72 mutants that have a base pair bind to EF-Tu-GTP. The relative affinity of T1 and G72 mutants for EF-Tu-GTP parallels their activity in elongation (Figs. 2–4). The relative affinity of the G72 mutant Met-tRNA for EF-Tu-GTP is lower by a factor of 10 than that of elongator Met-tRNA<sup>Met</sup>. However, it is comparable to that of some other E. coli aminocyl-tRNAs such as Val-tRNA, Leu-tRNA, Lys-tRNA, etc. (12).

Mutations in the GGGCCC sequence conserved in the anticodon stem of initiator tRNAs have little effect on their binding to EF-Tu-GTP. As expected from other work, mutant fMet-tRNAs in which the amino group of methionine is blocked by a formyl group also do not bind EF-Tu-GTP (22).

**DISCUSSION**

We have shown that a single base change enables the E. coli initiator tRNA to act as an elongator. The T1 and the G72 single mutations, which generate a Watson–Crick base pair at the end of the acceptor stem (Fig. 1), enable the E. coli initiator to act as an elongator, whereas the double mutant T1G72, which does not have such a Watson–Crick base pair, is inactive in elongation (Fig. 2). Thus, the absence of a Watson–Crick base pair at the end of the acceptor stem is one of the main features that prevents E. coli and most likely all prokaryotic initiator tRNAs from acting as elongators in protein synthesis. Schulman and Pelka (23) have shown that the same feature also accounts for the unique resistance (5) of E. coli fMet-tRNA toward peptidyl-tRNA hydrolase. Thus, the absence of a Watson–Crick base pair at the end of the acceptor stem in E. coli initiator tRNA explains at least two of its biological properties.

Of the two mutations that generate a Watson–Crick base pair at the end of the acceptor stem, the G72 mutant is much more active as an elongator than the T1 mutant. This is also reflected in the relative affinity of the Met-tRNAs derived from these two mutants for EF-Tu-GTP (G72 > T1 > T1G72 > wild-type tRNA). Thus, the mutant tRNAs are active in elongation primarily because they bind EF-Tu-GTP, in contrast to the wild-type tRNA, which does not. In this connection it should be noted that mutant tRNAs corresponding to our T1 and G72 mutants have been obtained previously. Schulman and co-workers (22) isolated E. coli initiator tRNA carrying a C1 → U1 change as among the products of sodium bisulfite reaction on the tRNA and showed that this tRNA bound EF-Tu-GTP, although the binding was weak compared to other aminocyl-tRNAs. Fischer et al. (24) obtained tRNA with A72 → G72 change by enzymatic manipulation of the tRNA and showed that this tRNA bound EF-Tu-GTP "strongly." Neither group, however, examined the subsequent binding of these tRNAs to the ribosomal A site nor tested their function as elongators in protein synthesis.

There is no simple explanation for the observation that G72 mutant tRNA binds better to EF-Tu-GTP than the T1 mutant. Since the presence of a Watson–Crick base pair at the end of the acceptor stem is considered necessary for binding of aminocyl-tRNAs to EF-Tu-GTP, a simple possibility is that this region of the molecule "breathes" more readily in the T1 mutant, which has a U-A base pair, than in the G72 mutant, which has a C-G base pair. Studies on interaction of T1, G72, and T1G72 mutant Met-tRNAs with Met-tRNA<sup>Met</sup>-formylase (unpublished work) suggest that there are differences in structure of the acceptor stem between the T1 and G72 mutants. This does not, however, explain the observation that Gln-tRNA, which has a U-A base pair at the end of the acceptor stem, is among aminocyl-tRNAs with the highest affinity for EF-Tu-GTP (12).

Our results that the T1, G72, T1T2A71, and T2A71G72 mutants, which retain the GGGCCC sequence in the anticodon stem conserved in initiator tRNAs, bind to ribosomal A site and are active in elongation do not necessarily conflict with the prevailing notion that the role of the GGGCCC sequence is to endow the anticodon loop of initiator tRNA a unique conformation, which is important for recognizing the initiator tRNA to the ribosomal P site during initiation (25).

It is possible that though the anticodon loop conformation of initiator tRNAs is "tailored" to fit into the ribosomal P site, it could also fit into the ribosomal A site, although perhaps less well than elongator tRNAs do. Our results agree with those of Yarus and coworkers (26), who introduced the GGGCCC sequence in the anticodon stem of Su7 amber suppressor tRNA and showed that the mutant tRNA was still an efficient amber suppressor in E. coli.

Although the mutant with changes in the GGGCCC sequence of E. coli initiator tRNA is in itself not active in elongation (Fig. 3), introduction of this mutation either in the T1 or the G72 mutant increases the activity of both mutants in elongation. This occurs without any significant increase in the affinity of the respective mutants for EF-Tu-GTP (Table 1). There are two possible explanations for this result. One is that the mutation in the GGGCCC sequence in the anticodon stem changes the conformation of the anticodon loop into that of a typical elongator tRNA such that the tRNA now fits better into the ribosomal A site. We showed previously that a conformational change of the anticodon loop does indeed occur with this mutant (11). The other possible explanation is that the changes that we have introduced in the anticodon stem sequence provide more favorable contacts between the anticodon stem and protein and/or RNA components of the ribosomal A site. The latter explanation corresponds to the "extended anticodon hypothesis" of Raftery and Yarus (27). The limited number of mutants in the anticodon stem that we have studied do not allow a choice among these possibilities.

Introduction of an additional T2A71 mutation into either a T1 or a G72 mutant also increases the activity of the latter mutants in elongation. It is not known whether this is due to a slightly higher affinity for EF-Tu-GTP of the T1T2A71 and T2A71G72 mutants over T1 and G72 mutants, respectively (data not shown), or whether introduction of the T2A71...
mutation provides more favorable contacts for the mutant initiator tRNAs near the CCA end and amino acid-binding region of the ribosomal A site.

Finally, although the T1 and the G72 single-base mutants are active in elongation in vitro, we do not know whether either of these mutants will act as elongators in vivo. However, of these two mutants, the G72 mutant is more likely to do so for the following reasons. First, affinity of the T1 mutant for EF-Tu-GTP is substantially lower than that of the G72 mutant (Table 1). Second, although the T1 mutant Met-tRNA is an excellent substrate for E. coli Met-tRNA transformylase (Ref. 28; unpublished observations), the G72 mutant is a poor substrate for this enzyme. This would mean that in vivo most of the T1 mutant will be present not as Met-tRNA but as fMet-tRNA, which (i) does not bind EF-Tu-GTP and (ii) cannot participate in elongation. Because of its low affinity for EF-Tu-GTP, the small amounts of T1 mutant Met-tRNA present in vivo are unlikely to act as an elongator, to any significant extent. In contrast, most of the G72 mutant will be present as Met-tRNA and, since it has a fairly high affinity for EF-Tu-GTP, will most likely act as an elongator in vivo. Additionally, since formation of the initiator Met-tRNA is important for initiation, the G72 mutant is less likely to act as an initiator in vivo to any significant extent. Thus, the G72 mutation may, in effect, be considered a mutation that has switched the function of a tRNA from initiation to elongation.

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