Suppression of Amber Codons in Vivo as Evidence That Mutants Derived from *Escherichia coli* Initiator tRNA Can Act at the Step of Elongation in Protein Synthesis*

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The absence of a Watson-Crick base pair at the end of the amino acid acceptor stem is one of the features which distinguishes prokaryotic initiator tRNAs as a class from all other tRNAs. We show that this structural feature prevents *Escherichia coli* initiator tRNA from acting as an elongator in protein synthesis in vivo. We generated a mutant of *E. coli* initiator tRNA in which the anticodon sequence is changed from CAU to CUA (the T35A36 mutant). This mutant tRNA has the potential to read the amber termination codon UAG. We then coupled this mutation to others which change the C1-A72 mismatch at the end of the acceptor stem to either a U1:A72 base pair (T1 mutant) or a C1:G72 base pair (G72 mutant). Transformation of *E. coli* CA274 (HfrC Su lacZ125am trpEam) with multicopy plasmids carrying the mutant initiator tRNA genes show that mutant tRNAs carrying changes in both the anticodon sequence and the acceptor stem suppress amber codons in vivo, whereas mutant tRNA with changes in the anticodon sequence alone does not. Mutant tRNAs with the above anticodon sequence change are aminoacylated with glutamine in vitro. Measurement of kinetic parameters for aminoacylation by *E. coli* glutaminyl-tRNA synthetase show that both the nature of the base pair at the end of the acceptor stem and the presence or absence of a base pair at this position can affect aminoacylation kinetics.

We discuss the implications of this result on recognition of tRNAs by *E. coli* glutaminyl-tRNA synthetase.

Every prokaryotic initiator tRNA sequenced has a 5'-terminal nucleotide which is not base-paired to the corresponding nucleotide on the 3' side of the amino acid acceptor stem (1). In *Escherichia coli* initiator tRNA, the 5'-terminal nucleotide is C (C1), and the corresponding 3' nucleotide is A (A72). Recently, we showed that a single base mutation which generated either a U1:A72 base pair (T1 mutant) or a C1:G72 base pair (G72 mutant) enabled these tRNAs to act as elongators, in vitro, whereas the double mutant (C1A72 → U1G72) did not (2). The G72 mutant was more active than the T1 mutant, and this was ascribed to a tighter binding of the G72 mutant to elongation factor EF-Tu compared to the T1 mutant. However, it was not known whether these mutants would also be active in elongation in vivo.

The most direct approach to demonstrate in vivo activity of mutant initiator methionine tRNAs in elongation is to isolate *E. coli* strains in which elongator tRNA<sup>Met</sup> synthesis or function is conditionally defective and to show that this defect could be complemented by mutants derived from initiator tRNAs. Isolation of such strains is, however, difficult since there are two genes for elongator tRNA<sup>Met</sup>, and both of these genes are part of a multiple tRNA gene operon in which one of these elongator tRNA<sup>Met</sup> genes is the promoter proximal gene for the entire operon (3, 4). Thus, conditional inactivation of the promoter or disruption of tRNA<sup>Met</sup> genes would affect expression or processing of transcripts of other tRNA genes which are downstream and some of which are essential for growth (3, 4). An alternative approach is to generate a nonsense suppressor derived from the initiator tRNA. Nonsense suppression, by definition, is a property of elongator tRNAs since suppressors insert amino acids internal to the polypeptide chain. In this work, we have changed the anticodon sequence of *E. coli* initiator tRNA from CAU into CUA, combined the anticodon mutation with mutations which introduce a base pair at the end of the acceptor stem, and examined whether these mutants suppress amber codons in vivo. Since mutants carrying the anticodon sequence change from CAU to CUA are aminoacylated with glutamine (5), we have also analyzed the kinetic parameters for aminoacylation of the partially purified mutant *E. coli* initiator tRNAs (6) using purified *E. coli* GlnR synthetase. We show that both the presence or absence of a base pair at the end of the acceptor stem and the nature of this base pair can affect kinetic parameters of aminoacylation, and we discuss the implications of this result concerning recognition of tRNAs by *E. coli* GlnR synthetase.

**MATERIALS AND METHODS**

Isolation of Mutant tRNAs—Mutagenesis of *E. coli* initiator tRNA<sup>Met</sup> gene and isolation of mutant tRNAs were as described (2, 6).

**In Vivo Activity of Mutant tRNAs in Suppression**—The mutant tRNA genes were subcloned into the Pet1-EcoRI site of pBR322 and used to transform *E. coli* CA274 (HfrC Su lacZ125am trpEam) (7). Transformants were scored for suppression of amber codons using the following criteria: formation of purple colonies on MacConkey lactose plates, formation of blue colonies on IPTG/X-Gal plates, and ability of transformants to grow on minimal plates lacking tryptophan and with lactose as the sole carbon source (8).

**Assay for β-Galactosidase Activity in *E. coli* CA274 Transformants—**β-Galactosidase was measured according to Miller (9). Briefly, *E. coli* cells were grown in 2YT media containing tetracycline and 1 mM IPTG to an A<sub>600</sub> of 0.6 to 0.8. After centrifugation, cell pellets were resuspended into one-tenth volume of lacZ buffer and chilled on ice. An aliquot of cell suspension was diluted into 1 ml of lacZ buffer, and 1 drop of CHCl<sub>3</sub> and 1 drop of 0.1% SDS were added and vortexed.
briefly for lysis. The amount of cell suspension used varied for the different transformants, more for cells producing low levels of \( \beta \)-galactosidase and less for those producing high levels of \( \beta \)-galactosidase. After 5 min at 28 °C, 0.2 ml of O-nitrophenyl-\( \beta \)-galactopyranoside solution was added, and the mixture was incubated at the same temperature, following which 0.5 ml of Na\(_2\)CO\(_3\) was added. The sample was then chilled on ice and centrifuged. Supernatant was taken out for measurement of absorbance at 420 nm. The \( \beta \)-galactosidase activity units given are in hours.

**Aminoclaylation of Mutant tRNAs by E. coli GlnR Synthetase**—

Assay conditions for aminoclaylation by GlnR synthetase were essentially as described (3). The incubation mixture (33 \( \mu \)l) contained 100 mM sodium cacodylate, pH 7.1, 10 mM magnesium acetate, 2 mM ATP, 310 \( \mu \)M \[^3\text{H}\]glutamine, and varying amounts of either E. coli tRNA\(^{\text{Am}}\) or tRNA\(^{\text{Sm}}\) mutants which were isolated by gel electrophoresis. Incubation was for 2.5 min at 37 °C in the presence of appropriate dilutions of purified E. coli GlnR synthetase (gift from John Perona, Yale University). An aliquot (18 \( \mu \)l) from each reaction was used for measurement of acid-insoluble radioactivity.

**RESULTS**

**Mutants of E. coli Initiator tRNA**—The mutants used in this work, T35A36, T1/T35A36, T35A36/G72, T35A36/G72G73, and G72G73, were generated in M13mp8 using previously described procedures (2, 6). These mutants contain changes at either the anticodon sequence (T35A36), the acceptor stem (G72G73), or both (T1/T35A36, T35A36/G72, and T35A36/G72G73) (Fig. 1).

**Activity of Mutant tRNA in Vivo in Suppression of Amber Codons**—Analysis of CA274 (HfrC Su*-lacZam trpEam) transformants carrying the mutant tRNA genes in multicopy plasmids showed that mutants with changes in either the anticodon sequence alone (T35A36 mutant) or in the acceptor stem alone (G72G73 mutant) were inactive in suppression, whereas those with changes in both regions (the T1/T35A36, T35A36/G72, and T35A36/G72G73 mutants) were all active in suppressing the amber mutant alleles in lacZ\(125\)am and in

![Sequence of E. coli formylmethionine initiator tRNA (tRNA\(^{\text{Am}}\)) in cloverleaf form and the sites of mutation studied in this report as indicated by arrows.](image)

\(A\) to \(G73\)
\(\Delta36\)

**Kinetic Parameters in Aminoclaylation of Mutant Initiator tRNAs by E. coli GlnR Synthetase**—Table III compares the kinetic parameters in aminoclaylation of the various mutant initiator tRNAs to those for E. coli tRNA\(^{\text{Am}}\). Of the mutants used in this study, the best substrate for E. coli GlnR synthetase is the T35A36 mutant with the anticodon sequence changed from CAU to CUA. The \(V_{\max}/K_m^{\text{Am}}\) for this mutant is about 20-fold lower than that for E. coli tRNA\(^{\text{Am}}\) (Table II). This value agrees reasonably well with an approximately 400-fold difference previously reported by Schulman and Pelka (5) between the corresponding tRNA\(^{\text{Am}}\)-mutant and tRNA\(^{\text{Am}}\). Interestingly, the effects of introducing changes in the acceptor stem on the T35A36 mutant initiator tRNA are quite variable. Introduction of a U1A72 base pair at the end
Recognition of tRNAs by E. coli GlnR Synthetase

TABLE I
Suppressor phenotype of E. coli CA274 transformants carrying the various mutant initiator tRNA genes or the Su'2 tRNA^{Gln} gene

| Plate Conditions | CA274(Su') | Mutants  |
|------------------|------------|---------|
| A. Lactose/minimal | T35A36 | T35A36/T1 | T35A36/G72 | T35A36/G72G73 | G72G73 | CA274/Su'2 |
| B. MacConkey/lactose + IPTG | + | + | + | ++ | + | ++ |
| C. IPTG + X-Gal | + | + | + | + | + | +++ |

FIG. 2. Growth of E. coli CA274 (HfrC Su' lacZ125am, trpEam) transformed with plasmids carrying mutant initiator tRNA genes or Su'2 tRNA^{Gln} on minimal plate containing lactose as the sole carbon source and lacking tryptophan, pLAR220 which carries the E. coli Su'2 tRNA^{Gln} gene under control of lacUV5 promoter was isolated from MY544 (gift from M. Yarus, University of Colorado) and was used to transform CA274. Plate shown here has been incubated at 37°C for 2½ days. Differences in growth rate were observed during incubation among various mutants, which are summarized in Table I.

of the acceptor stem, which is present in both of the E. coli tRNA^{Gln} isoacceptor species, does not make the T1/T35A36 mutant tRNA a better substrate for E. coli GlnR synthetase than the T35A36 mutant; the V_{max}/K_{m}^{wt} for the T1/T35A36 mutant is, actually, about 3-fold lower than for the T35A36 mutant. Introduction of a C1:G72 base pair at the end of the acceptor stem, however, has a significantly deleterious effect, the V_{max}/K_{m}^{wt} for the T35A36/G72 mutant being approximately 30-fold lower than for the T35A36 mutant. These results suggest that in the context of E. coli tRNA^{Met} sequence, E. coli GlnR synthetase prefers those tRNA substrates which either lack a base pair or have a U:A base pair at the end of the acceptor stem over those which have a C:G base pair. Finally, the approximately 5-fold higher V_{max}/K_{m}^{wt} for the T35A36/G72G73 mutant relative to the T35A36/G72 mutant suggests that mutant tRNA with the anticodon sequence change alone is inactive as an amber suppressor because of formylation of the T35A36/G72G73 mutant with the C1/G72 base pair was much more active in elongation than the T1 mutant with the U1:A72 base pair alone. This result is in agreement with the notion that G72 may be one of the sites of interaction between E. coli GlnR synthetase and the tRNA substrate. The nucleotide G73 is present in both of the E. coli tRNA^{Gln} isoacceptor species and in a Su' mutant derived from E. coli tRNA^{ Tyr} which is a substrate for E. coli GlnR synthetase (13). In addition, a mutant of E. coli Su'3 tRNA^{ Tyr} with G73 instead of A73 inserts glutamine instead of tyrosine (14, 15). The finding that the V_{max}/K_{m}^{wt} for the T35A36 mutant is higher than that for the T35A36/G72G73 mutant suggests, however, that while G73 may provide a site of interaction between E. coli GlnR synthetase and the tRNA substrate, this interaction is not absolutely essential for aminocacylation of a tRNA by E. coli GlnR synthetase.

DISCUSSION

We have shown that the absence of a base pair at the end of the acceptor stem, which is a hallmark of all prokaryotic initiator tRNAs, prevents these tRNAs from acting in elongation in vivo. We changed the anticodon sequence of E. coli GlnR synthetase such that the tRNA has the potential capability of reading the amber termination codon UAG and combined this with mutations which generate either a U:A or a C:G base pair at the end of the acceptor stem. The mutant tRNA with the anticodon sequence change alone is inactive in amber suppression in vivo, whereas mutants carrying both the anticodon sequence change and the acceptor stem sequence change are active.

The inactivity of the T35A36 mutant tRNA^{Met} as an amber suppressor differs from previous results demonstrating in vivo suppression by mutants derived from E. coli Su'3 tRNAs which lack a Watson-Crick base pair at the end of the acceptor stem (7). There are several possible explanations for this discrepancy. 1) The T35A36 mutant tRNA^{Met} is prevented from acting as a suppressor because of formylation of the T35A36 Gln-tRNA^{Met} in vivo to fGln-tRNA^{Met}. This is unlikely since the T1 mutant tRNA^{Met}, which is almost as good a substrate for Met-tRNA formylase in vitro as the wild-type tRNA^{Met} is active as an amber suppressor (Table I and Fig. 2). 2) The T35A36 mutant tRNA^{Met} is an extremely weak suppressor, and the criteria we have used for amber suppression (Table I) is unable to detect such suppressors. 3) Mutants of E. coli tRNA^{ Tyr} which lack a base pair at the end of the acceptor stem bind EF-Tu and the ribosomal A site well enough to suppress certain amber codons, whereas mutants of tRNA^{Met} do not. In other words, whether a tRNA lacking a Watson-Crick base pair at the end of the acceptor stem binds to the ribosomal A site or not depends also on the context of the tRNA sequence.

The relative activity of the various tRNA^{Met} mutants in suppression in vivo is different from that of the corresponding mutants in elongation in vitro (2). In vivo, the T1/T35A36 and T35A36/G72 mutants which carry, respectively, a U1:A72 and C1:G72 base pair at the end of the acceptor stem have about equal activity. In vitro assays showed previously that the G72 mutant with the C1:G72 base pair was much more active in elongation than the T1 mutant with the U1:A72 base pair. In itself, this discrepancy is not surprising. First, the in vivo activity of a mutant tRNA^{Met} in elongation depends upon several factors: amounts of mutant tRNA made, extent of aminocacylation, affinity of the aminoacyl-tRNA for EF-Tu, affinity for the A site on the ribosome, formulation of the aminoacyl-tRNA, etc. In contrast, for analysis of in vitro activity of a mutant tRNA^{Met} in elongation, the tRNAs are

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3 C.-P. Lee, B. Seong, and U. RajBhandary, unpublished observations.
provided as aminocyl-tRNAs, and assays are carried out in the absence of formyltetrahydrofolate. Thus, the activity of a mutant tRNA in elongation in vitro is simply a function of its affinity for EF-Tu and for the ribosomal A site. Second, while the mutants used for studying in vitro elongation activity contained only changes in the acceptor stem and are aminocylated with methionine, mutants used for assay of in vivo elongation activity (i.e. suppression) contained changes in both the anticodon sequence and the acceptor stem and are aminocylated not with methionine but with glutamine (5). Furthermore, the kinetic parameters for aminocylatation with glutamine are different for the various mutants (Table III). Thus, in vivo levels of Gln-tRNA\textsuperscript{Met} for the various mutants could vary widely. This possibility could explain the fact that the efficiency of amber suppression in cells carrying the T1/T35A36 and the T35A36/G72 mutant tRNA\textsuperscript{Met} genes are about equal. The T35A36/G72 mutant tRNA may have a higher affinity for EF-Tu than the T1/T35A36 tRNA (2), but this increased affinity for EF-Tu could be compensated for by a lower concentration of Gln-tRNA\textsuperscript{Met} corresponding to the T35A36/G72 mutant compared to the T1/T35A36 mutant. Similarly, an explanation for the higher efficiency of suppression with the T35A36/G727G2 mutant tRNA over the T35A36/G727G2 mutant tRNA is that the former is a better substrate for E. coli GlnR synthetase. Consequently, the steady state concentration in vivo of Gln-tRNA\textsuperscript{Met} corresponding to the T35A36/G727G3 mutant is likely to be higher than that of the T35A36/G727G2 mutant.

Results on kinetic parameters for aminocylatation of the mutant tRNAs with glutamine provide insights into how E. coli GlnR synthetase might interact with its tRNA substrate. The most surprising result is that a mutation which generates a U1:A72 base pair conserved in all eubacterial, chloroplast, and mitochondrial glutamine tRNAs does not result in an increase in the $V_{max}/K_{m}$ for the T35A36 mutant tRNA with a C1:A72 mismatch at the end of the acceptor stem is as good, if not a better substrate, as the T1/T35A36 mutant with a U1:A72 base pair. The T35A36/G727G2 mutant with a C1:A72 base pair is, on the other hand, a much poorer substrate. Thus, the U1:A72 base pair conserved in glutamine tRNAs may be there not because GlnR synthetase contacts specific functional groups in a U:A base pair, but is there to provide structural flexibility at the end of the acceptor stem. The structural flexibility provided by a weak base pair such as U1:A72 or a mismatch may be needed for GlnR synthetase to interact with G73 or for the tRNA-CCA end to fit into the catalytic pocket of GlnR synthetase or both. The occurrence of U1:A72 base pair in eubacterial, mitochondrial, and chloroplast tRNA\textsuperscript{Met} instead of no base pair, as in E. coli tRNA\textsuperscript{Met}, may be viewed as a compromise between the need for a base pair at this position for binding to EF-Tu and requirements for E. coli GlnR synthetase that this base pair be relatively weak compared to other base pairs.

The hypothesis that structural flexibility at the end of the acceptor stem may be important in aminoacylation of tRNA by E. coli GlnR synthetase has been proposed before (7, 19), although the absence of data on kinetic parameters for aminoacylation, and is now supported by analysis of mutants derived from several different E. coli tRNAs which can be aminoacylated by E. coli GlnR synthetase. 1) Three of the four mutants derived from E. coli Su+7 tRNA\textsuperscript{Met} which inserts glutamine in vivo, have a disruption in either the first or the second base pair in the acceptor stem (7). 2) The Su+7 amber suppressor derived from tRNA\textsuperscript{Met} by an anticodon sequence change from CCA to CU3 inserts glutamine in vivo. This tRNA has A1:U72, a “weak” base pair, and not G:C or C:G base pair at this position (13). 3) Mutants derived from E. coli Su+1 tRNA\textsuperscript{Met} which insert glutamine in vivo have recently been generated by site-specific mutagenesis (17). One of the changes replaced the G1:C72 base pair by a U1:A72 base pair. 4) Finally, a mutant tRNA derived from E. coli tRNA\textsuperscript{Met} which inserts glutamine in vivo has also been generated recently (18). This tRNA differs from other tRNAs aminoacylated with glutamine in that it has a G1:C72 base pair at the end of the acceptor stem. Interestingly, the mutation that resulted in its aminoacylation with glutamine in vivo was the change of G3:C70 in the acceptor stem to G3:U70. Presumably, the G:U base pair destabilizes the acceptor stem sufficiently to provide the structural flexibility needed for aminoacylation by E. coli GlnR synthetase. These results taken together support strongly the hypothesis that for aminoacylation of a tRNA by E. coli GlnR synthetase, the exact nature of the base pair at the end of the acceptor stem is not as crucial as the fact that the base pair be such as to provide structural flexibility in this region of the molecule.

The possible importance of structural flexibility in the acceptor stem in aminoacylation by tRNA by E. coli GlnR synthetase provides a second example in which structural flexibility could play an important role in interactions between tRNA and proteins. Studies on formylation of E. coli tRNA\textsuperscript{Met} mutants (19) have shown that tRNAs with C1:A72 or U1:G72 mismatches, or a U1:A72 base pair at the end of the acceptor stem, are all almost equally good substrates for Met-tRNA formylase, whereas tRNA with a C1:G72 base pair is a very poor substrate. It would, therefore, not be surprising if further work reveals other examples in which structural flexibility in the region proximal to the -CCA end of tRNA is important in interaction between tRNA and aminoacyl-tRNA synthetases or other proteins.

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