Important Role of the Amino Acid Attached to tRNA in Formylation and in Initiation of Protein Synthesis in Escherichia coli*

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In attempts to convert an elongator tRNA to an initiator tRNA, we previously generated a mutant elongator methionine tRNA carrying an anticodon sequence change from CAU to CUA along with the two features important for activity of Escherichia coli initiator tRNA in initiation. This mutant tRNA (Mi:2 tRNA) was active in initiation in vivo but only when aminoacylated with methionine by overproduction of methionyl-tRNA synthetase. Here we show that the Mi:2 tRNA is normally aminoacylated in vivo with lysine and that the tRNA aminoacylated with lysine is a very poor substrate for formylation compared with the same tRNA aminoacylated with methionine. By introducing further changes at base pairs 4:69 and 5:68 in the acceptor stem of the Mi:2 tRNA to those found in the E. coli initiator tRNA, we show that change of the U4:A69 base pair to G4:C69 and overproduction of lysyl-tRNA synthetase and methionyl-tRNA transformylase results in partial formylation of the mutant tRNA and activity of the formyllysyl-tRNAs in initiation of protein synthesis. Thus, the G4:C69 base pair contributes toward formylation of the tRNA and protein synthesis in E. coli can be initiated with formyllysine. We also discuss the implications of these and other results on recognition of tRNAs by E. coli lysyl-tRNA synthetase and on competition in cells among aminoacyl-tRNA synthetases.

Two species of methionine tRNAs are present in all organisms. The initiator methionine tRNA is used for initiation of protein synthesis, whereas the elongator methionine tRNA is used for insertion of methionine into internal peptidic linkages (1–3). In eubacteria, mitochondria, and chloroplasts, the initiator tRNA is used as formylmethionyl-tRNA (fMet-tRNA), whereas in the cytoplasm of eukaryotes it is used as methionyl-tRNA (Met-tRNA). In Escherichia coli, formylation of the initiator Met-tRNA is important for initiation of protein synthesis (5–7). This reaction is catalyzed by the enzyme methionyl-tRNA transformylase (8).

The distinct roles of the two species of methionine tRNAs in protein synthesis are a result of their different structures. In E. coli, the C13A172 mismatch at the end of the acceptor stem along with G2:C71 and C3:G70 base pairs are important for formylation of the tRNA (9–11), and the three consecutive G:C base pairs in the anticodon stem are important for binding of the fMet-tRNA to the ribosomal P site (12). However, transplanting these features into the E. coli elongator methionine tRNA also carrying an anticodon sequence change from CAU to CUA did not generate a tRNA that was itself active in initiation. The tRNA thus generated, Mi:2 tRNA, could initiate protein synthesis only in cells overproducing MetRS (13). The reason for this was that the mutant tRNA was formylated when aminoacylated with methionine but not with the amino acid normally attached to the Mi:2 tRNA. In contrast, transplanting the same features into E. coli glutamine tRNA resulted in a tRNA, Qi:2 tRNA, which was fairly active in initiation (13). A possible explanation of these results is that unlike the Qi:2 tRNA, which is most likely aminoacylated with glutamine, the Mi:2 tRNA is aminoacylated with an amino acid that negatively affects its formylation and/or some subsequent step in initiation of protein synthesis. A further possibility is that E. coli tRNA^Gln and tRNA^Met have in common some sequence and/or structural feature that contributes toward initiation and that is lacking in the elongator tRNA^Met.

In this paper, we show that the Mi:2 tRNA is aminoacylated with lysine in vivo and that when aminoacylated with lysine, it is a very poor substrate for formylation. We have introduced further changes at base pairs 4:69 and 5:68 in the acceptor stem of the Mi:2 tRNA to G:C base pairs present in E. coli tRNA^Met and in tRNA^Gln and studied the effect of these changes on aminoacylation of the tRNA with LysRS and on formylation. We show that the mutant tRNAs with the G4:C69 base pair (Mi:2/4 and Mi:2/4, 5 tRNA) are now better substrates for formylation. Overproduction of LysRS and MTF leads to partial formylation of these mutant tRNAs and the formyllysyl-tRNAs are active in initiation of protein synthesis. These results highlight the role of the amino acid attached to the tRNA in formylation and thereby in initiation of protein synthesis and the role of the G4:C69 base pair in recognition of the tRNA by MTF, specifically in the sequence context of the E. coli tRNA^Met. They also show that protein synthesis in E. coli can be initiated with formyllysine.

We have also studied the effects of overproduction of glutamyl-tRNA synthetase (data not shown), MetRS, and MetRS and MTF on aminoacylation of the mutant tRNAs and on their activities in formylation and initiation. We discuss the implications of these and other results on recognition of tRNAs by LysRS and on competition in cells between glutamyl-tRNA synthetase, LysRS, and MetRS for tRNAs.

MATERIALS AND METHODS

E. coli Strains and Plasmids—E. coli strain CA274 (HfrHLac-ZamTrpEam) was used as the host strain for analysis of the activity of the various mutant tRNA genes. The tRNA genes were cloned into pRSVPcATam1.2.5 and pRSVPcATam5–6H plasmids as described (14, 15). The genes for MetRS, LysRS, and MTF were cloned into pACYC184 (16), a derivative of pACYC184.

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§ The abbreviations used are: tMet-tRNA, formylmethionyl-tRNA; Met-tRNA, methionyl-tRNA; MetRS, methionyl-tRNA synthetase; MTF, methionyl-tRNA transformylase; LysRS, lysyl-tRNA synthetase; CAT, chloramphenicol acetyltransferase.
Assays for Chloramphenicol Acetyltransferase (CAT) Activity in Extracts—CA274 containing the pRSVPcatTam1.25 and the pACD vectors were grown at 37 °C overnight in 2 × YT medium containing ampicillin (100 mg/ml) and tetracycline (12.5 mg/ml). The overnight culture was diluted 1–20- to 1–200-fold in 2 × YT medium containing ampicillin (50 mg/ml) and tetracycline (8 mg/ml) and was grown for about 37 °C to an optical density of 0.3–0.5. Cell extracts from 1.2 ml of the fresh culture were used for assays of CAT activity and β-lactamase activity (5). CAT activity was normalized to β-lactamase activity to eliminate the effect of any possible variations in copy number of the pRSVPcat plasmid. The relative CAT activity in cells carrying the U35A36 mutant of E. coli initiator tRNA was fixed at 100%.

Preparation and Amino-terminal Sequence Analysis of CAT Protein Carrying Six Histidine Residues—CAT protein with six histidine residues was purified by adsorption to nickel-chelate resin (X PRESS SYSTEM, Invitrogen Inc.) as described (15) except that native conditions were used for binding of the protein and washing of the resin. E. coli CA274 containing pRSVPcatTam5–6H/Mi:2 was grown overnight in 1 liter of 2xYT medium at 37 °C and harvested by centrifugation at 4 °C. The cell pellet was resuspended in 6.5 ml of native binding buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8) containing 0.1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was incubated on ice for 15 min, frozen and thawed three times, and centrifuged for 10 min at 4 °C. The supernatant was collected and mixed with nickel-chelate resin (3.6 ml) that was equilibrated in native binding buffer, pH 7.8. After incubation at 4 °C for 2 h with constant mixing, the resin was washed with native washing buffer (same as binding buffer except that pH was 6.0) until an A<sub>260</sub>/ml of 0.02 was obtained. The resin was further washed with native pH elution buffer (same as binding buffer, except that pH was 4.0) or native imidazole elution buffer (like native buffer except 0.3 M imidazole was included, pH 6.0). The bound proteins were eluted in Laemmli sample buffer containing 2% SDS, 0.062 M Tris-HCl, pH 6.8, and 14.4 mM β-mercaptoethanol. Five fractions (1 ml each) were collected, and the fractions containing CAT protein were identified by immunoblot analysis using anti-CAT antibody and concentrated by methanol precipitation (16). The concentrated preciptate was renatured in coupled elution buffer (19) and electroblotted onto Nytran Blue One. Of the lanes containing ~1% of total protein applied was sliced off and used for immunodetection of CAT protein using anti CAT antibodies (5). The CAT protein band on the Coomassie-stained membrane was excised and used for sequencing (18).

Polyacrylamide Gel Electrophoresis and Northern Blot Analysis of tRNAs—Cells expressing the mutant tRNAs (approximately 3 ml) were grown to an A<sub>260</sub>/ml of 0.3–0.5 (see above), pelleted, resuspended in 0.3 M sodium acetate, pH 4.5, and 1 mM EDTA, and extracted with an equal volume of phenol equilibrated with the same buffer (19). The aqueous layer was transferred to another tube and extracted once more with phenol. The final aqueous layer was then mixed with three volumes of ethanol and left on dry ice for 30 min to precipitate the tRNAs. The tRNAs were dissolved in 10 mM sodium acetate, pH 4.5, and 1 mM EDTA. The tRNAs were separated by electrophoresis on an acid-urea gel (19) and electroblotted onto Nittran membrane, and the mutant tRNAs were detected by hybridization to a 125I-labeled oligonucleotide probe (5'-CCAGGGTTTGAAGCCCCTG-3') complementary to nucleotides 25–44 of the mutant elongator methionine tRNAs described in this work.

RESULTS

The Mi:2 tRNA Is Aminoacylated with Lysine in Vivo—In attempts to switch an elongator tRNA to an initiator tRNA, we had previously transplanted into E. coli tRNA<sup>Met</sup> several of the features important for activity of the E. coli initiator tRNA in initiation. These mutations were coupled to the anticodon sequence change from CAU to CUA to allow assessment of the initiation activity of the mutant tRNAs in vivo (13). Fig. 1 shows a clover leaf structure of one of the mutant tRNAs, the Mi:2 tRNA. This tRNA was active in initiation in vivo but only in cells overproducing MetRS. This is because the mutant tRNA was formylated when aminoacylated with methionine but not with the amino acid normally attached to the Mi:2 tRNA. In cells not overproducing MetRS, the Mi:2 tRNA was partially aminoacylated but not formylated (13). Although the identity of the amino acid attached to the Mi:2 tRNA was not established then, we considered the possibility that it was lysine or glutamine.

To determine the identity of the amino acid attached to the Mi:2 tRNA, we have taken advantage of the fact that although this mutant tRNA with a C1xA72 mismatch at the end of the acceptor stem has a much reduced activity in elongation, it can act as a weak suppressor of amber codons (20). The Mi:2 tRNA was, therefore, cloned into the plasmid vector (CATam5–6H) carrying a mutant chloramphenicol acetyltransferase reporter gene with a UAG amber codon at position 5 followed by six histidine codons. The CAT protein made was purified by affinity chromatography and subjected to amino-terminal sequence analysis (18). Results in Fig. 2 show that the amino acid inserted at the site of the UAG codon by the Mi:2 tRNA is lysine.

Introduction of Additional Mutations into the Mi:2 tRNA Gene—In contrast to the Mi:2 tRNA, which is not formylated in cells unless MetRS is overproduced, the Qb tRNA derived from tRNA<sup>CAT<sub>am</sub></sup> is essentially all formylated (13). Both mutant tRNAs contain the same nucleotides at positions 1 and 72 and base pairs 2:71 and 3:70 in the acceptor stem, which are important for formylation (9). The difference between the two tRNAs in the acceptor stem is the absence in the Mi:2 tRNA and the presence in the Qb tRNA of base pairs G4:C69 and G5:C68 found in the E. coli initiator tRNA. Other studies have also implicated a potential role for G4:C69 and G5:C68 base pairs on formylation (9) particularly in the context of an E. coli elongator tRNA<sup>Met</sup> sequence background (10).
observations, we introduced these G:C base pairs into the Mi:2 tRNA gene to investigate whether these mutant tRNAs would now be better substrates for formylation and hence be active in initiation without requiring the overproduction of MetRS. The new mutant tRNAs are designated as Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs (Fig. 1).

Activity of Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs in Initiation in Cells Overproducing MTF, LysRS, and Both MTF and LysRS—Previous work has shown that activity of mutant initiator tRNAs that are defective in initiation due to a block in formylation can be rescued by overproduction of the formylating enzyme MTF (16, 20). Therefore, we investigated the effect of overproduction of MTF, LysRS, and both MTF and LysRS on activity of the mutant tRNAs in initiation from a UAG initiation codon (5, 13, 14). Fig. 3 shows the vectors used to coexpress the CAT reporter gene and the mutant tRNAs (pRSvCATam1.2.5) and to overproduce the various enzymes (pACD).

Table I shows the results. Overproduction of MTF alone leads to a small increase in CAT activity with all of the mutant tRNAs. Overproduction of LysRS alone has no effect on CAT activity with any of the mutant tRNAs tested. However, overproduction of MTF and LysRS results in a substantial increase in CAT activity in cells expressing the Mi:2/4 and Mi:2/4,5 tRNAs but not in those expressing the Mi:2 and the Mi:2/5 tRNAs. Immunoblot analysis of extracts of cells expressing the mutant tRNAs and overproducing MTF and LysRS (Fig. 4) also confirms these results. A band corresponding to CAT protein is seen in extracts of cells carrying the Mi:2/4 and Mi:2/4,5 tRNAs but not the Mi:2 and Mi:2/5 tRNAs. All of the extracts contain β-lactamase, another protein encoded by the same pRSvCATam1.2.5 plasmid.

Aminoacylation and Formylation of the Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs in Cells Overproducing MTF, LysRS, and Both MTF and LysRS—Northern blot analysis of tRNAs isolated from cells carrying the various mutant tRNA genes indicates a clear correlation between activity of the mutant tRNAs in initiation and their formylation. Electrophoresis of tRNAs on acid urea polyacrylamide gels was used to separate various forms of the tRNA (deacylated tRNA, aminoacyl-tRNA, and formylaminoacyl-tRNA) from each other (19). The different forms of the tRNA were detected by Northern blot analysis using an oligonucleotide complementary to the anticodon stem and loop region of the tRNAs as the probe. Fig. 5 shows the results of such an analysis on tRNAs isolated from cells carrying the Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNA genes and overproducing MTF, LysRS, and both MTF and LysRS.

In cells not overproducing any of the enzymes (vector alone, lane 1 of Fig. 5, A–D), the mutant tRNAs are aminoacylated with lysine to varying degrees. When LysRS is overproduced, all the mutant tRNAs are essentially quantitatively aminoacylated with lysine (lanes 3 of Fig. 5, A–D). However, the Lys-tRNAs are not formylated. When both MTF and LysRS are overproduced, the Lys-tRNAs corresponding to the Mi:2/4 and Mi:2/4,5 tRNAs are now partially formylated. These are also the two tRNAs that showed clear activity in initiation in vivo under these conditions. There was no formylation of Lys-tRNAs corresponding to the Mi:2 and Mi:2/5 tRNAs in spite of the fact that the tRNAs are essentially quantitatively aminoacylated. These results suggest that the presence of a G4:C69 base pair makes the Lys-tRNAs better substrates for MTF, whereas the presence of a G5:C68 base pair has no such effect or a marginal effect at the most.

Although all of the above mutant tRNAs are aminoacylated with lysine in cells not overproducing any of the enzymes (lanes 1, Fig. 5, A–D), there are differences in the extent of aminoacylation among the mutant tRNAs. The relative intensity of the Lys-tRNA band compared with the uncharged tRNA band is clearly higher for the Mi:2/5 and Mi:2/4,5 tRNAs than for the other tRNAs. Table II provides a quantitative estimate of aminoacylation (19) of the four tRNAs with lysine using a PhosphorImager analysis of a Northern blot on a different batch of tRNAs. The results show that the presence of G4:C69 and
G5:C68 base pairs on the Mi:2 tRNA make the tRNAs better substrate for LysRS, the effect of the G5:C68 base pair being more pronounced than that of the G4:C69 base pair.

Activity of Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs in Initiation in Cells Overproducing MTF, MetRS, and both MTF and MetRS

We have also studied the effect of overproduction of MTF, MetRS, and both MTF and MetRS on the activity of the mutant tRNAs in initiation and in parallel their effect on the aminoacylation and formylation of the tRNAs in vivo. The results obtained with the mutant tRNAs aminoacylated with methionine are consistent with those obtained above with the tRNAs aminoacylated with lysine. Table III shows the results on activity of the mutant tRNAs in initiation. The data on the

Table I

| Proteins overproduced | Relative CAT activitya |  |
|-----------------------|-----------------------|---|
|                       | Mi:2 | Mi:2/4 | Mi:2/5 | Mi:2/4,5 |
| None                  | 0.4 ± 0.1b | 0.7 ± 0.1 | 0.9 ± 0.2 | 1.1 ± 0.1 |
| MTF                   | 2.6 ± 0.3 | 3.7 ± 1.0 | 4.8 ± 1.7 | 8.5 ± 2.7 |
| LysRS                 | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.0 | 0.8 ± 0.4 |
| LysRS and MTF         | 2.7 ± 1.2 | 56.8 ± 12.0 | 3.5 ± 1.2 | 64.9 ± 18.5 |

a The CAT activities are normalized to that of tRNA^fMet (CUA), which is set as 100%.
b Average of three separate assays on three individual extracts. All the other numbers are average of two to six individual assays.

Role of the Amino Acid Attached to Initiator tRNA in E. coli

G5:C68 base pairs on the Mi:2 tRNA make the tRNAs better substrate for LysRS, the effect of the G5:C68 base pair being more pronounced than that of the G4:C69 base pair.

Activity of Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs in Initiation in Cells Overproducing MTF, MetRS, and both MTF and MetRS—We have also studied the effect of overproduction of MTF, MetRS, and both MTF and MetRS on the activity of the mutant tRNAs in initiation and in parallel their effect on the aminoacylation and formylation of the tRNAs in vivo. The results obtained with the mutant tRNAs aminoacylated with methionine are consistent with those obtained above with the tRNAs aminoacylated with lysine. Table III shows the results on activity of the mutant tRNAs in initiation. The data on the effect of overproduction of MTF alone are the same as described above (Table I) and are reproduced here only for comparison. With the Mi:2 tRNA, overproduction of MetRS alone (21) increases CAT activity significantly as shown before (13). Overproduction of both MetRS and MTF leads to a further increase
in CAT activity of about 3-fold. This is most likely because more of the Mi:2 tRNA that is aminoacylated with methionine is now formylated (Fig. 6A, compare lanes 3 and 4). With the Mi:2 tRNA, CAT activity in cells carrying this tRNA and overproducing MetRS alone is about 2-fold higher than in cells carrying the Mi:2 tRNA; however, there is no further increase upon overproduction of MTF. This is consistent with the conclusion above that the presence of a G4:C69 base pair makes the Mi:2 tRNA a better substrate for MTF, such that essentially all of the tRNA that is aminoacylated with methionine in cells overproducing MetRS is now formylated (Fig. 6C, compare lanes 3 and 4). With the Mi:2 tRNA, the results are essentially the same as with the Mi:2 tRNA. With the Mi:2,4,5 tRNA, the effect of overproduction of MTF and MetRS or MetRS alone basically mirror those seen with the Mi:2 tRNA, except that CAT activities in cells carrying the Mi:2,4,5 tRNA is about half those with the Mi:2 tRNA. This is probably because this tRNA is the best substrate for LysRS among the four tRNAs tested (Table II). Consequently, in cells overproducing MetRS, less of the Mi:2,4,5 tRNA gets aminoacylated with methionine and subsequently formylated than the Mi:2/4 tRNA (Fig. 6, lanes 3 and 4). With the Mi:2/5 tRNA, the results are essentially the same as with the Mi:2 tRNA. With the Mi:2,4,5 tRNA, the effect of overproduction of MTF and MetRS or MetRS alone basically mirror those seen with the Mi:2/4 tRNA, except that CAT activities in cells carrying the Mi:2,4,5 tRNA is about half those with the Mi:2/4 tRNA. This is probably because this tRNA is the best substrate for LysRS among the four tRNAs tested (Table II). Consequently, in cells overproducing MetRS, less of the Mi:2,4,5 tRNA gets aminoacylated with methionine and subsequently formylated than the Mi:2/4 tRNA (Fig. 6, lanes C and D, compare the relative intensities of the band corresponding to fMet-tRNA to bands corresponding to formyllysyl-tRNAs and Lys-tRNA combined in lanes 3 and 4 of C to lanes 3 and 4 of D, respectively).

**DISCUSSION**

Recognition of Aminoacyl-tRNAs by MTF: Role of the Amino Acid and tRNA Sequence—This study has provided a clear indication of the important role of the amino acid attached to the tRNA in formylation and in initiation of protein synthesis in E. coli. We have shown that the Mi:2 mutant tRNA is aminoacylated with lysine in vivo and that the tRNA charged with lysine is an extremely poor substrate for formylation. For example, in cells overproducing LysRS, the mutant tRNA is essentially all aminoacylated with lysine, but the Lys-tRNA is not formylated (lane 3 of Fig. 5A and Table I). In contrast, in cells overproducing MetRS, a part of the tRNA is aminoacylated with methionine, and the Met-tRNA is now substantially formylated (lane 3 of Fig. 6A; see also Fig. 4 of Ref. 13). These results show that MTF has a very strong preference for methionine over lysine. Previous work on a mutant of E. coli initiator tRNA aminoacylated with glutamine (9, 22) and the initiator tRNA misaminoacylated with phenylalanine and valine (23) suggested a certain hierarchy in the preference of MTF for amino acids attached to tRNA in the following order: Met > Gln > Phe > Val. The current work allows us to add Lys to this list and indicates that it is among the least preferred of the amino acids for MTF.

The amino acid moiety also plays a role in at least one later step in initiation of protein synthesis subsequent to formylation of the tRNA. Previous studies have shown that the U35A36 mutant of E. coli initiator tRNA is a better initiator when it carries fMet than when it carries fGln (21). This suggests that some component of the translational machinery that interacts directly with the initiator tRNA prefers fMet over fGln. Because the initiation factor IF2 is known to interact with the acceptor stem of the tRNA (24) and requires an N-acetylated amino acid for binding (25), the affinity of IF2 for the formylamino acid moiety could be another factor that determines the activity of a tRNA in initiation. Some evidence for this possibility comes from the finding that activity of the U35A36 mutant initiator tRNA carrying fGln is increased greatly upon overproduction of IF2 (16). Thus, the absolute conservation of methionine as the initiating amino acid in all organisms could be due to the fact that the amino acid is inspected at two distinct steps in the initiation pathway with methionine being the most preferred amino acid at each of these steps. In eukaryotes where the initiator tRNA is not formylated, there are indications that the initiation factor eIF2 may have an absolute requirement for methionine attached to the initiator tRNA (26).

The introduction of further changes in base pairs 4:69 and 5:68 in the acceptor stem of the Mi:2 tRNA to G:C base pairs found at these positions in the E. coli initiator tRNA has shown that the presence of the G4:C69 base pair makes the tRNA a better substrate for MTF. Thus, in cells overproducing LysRS and MTF, the Mi:2 tRNA is aminoacylated but is not formylated. Consequently, it has very little activity in initiation. In contrast, a substantial fraction of the Mi:2/4 and Mi:2/4,5 tRNAs carrying the G4:C69 base pair is now formylated in vivo (lane 4 of Fig. 5, C and D), and these tRNAs show significant activity in initiation. The Mi:2/5 tRNA behaves essentially similarly to the Mi:2 tRNA, suggesting that the presence of the G5:C68 base pair does not have the same effect as the G4:C69 base pair; there is no detectable formylation of Mi:2/5 tRNA in cells overproducing LysRS and MTF (lane 4 of Fig. 5B). Besides

**Table II**

| tRNA     | Aminoacylation |
|----------|----------------|
| Mi:2     | 43             |
| Mi:2/4   | 46             |
| Mi:2/5   | 52             |
| Mi:2/4,5 | 58             |

**Table III**

| Proteins overproduced | Relative CAT activity* |
|-----------------------|------------------------|
|                       | Mi:2 | Mi:2/4 | Mi:2/5 | Mi:2/4,5 |
| None                  | 0.4  ± 0.1 | 0.7  ± 0.1 | 0.3  ± 0.1 | 1.1  ± 0.1 |
| MTF                   | 2.6  ± 0.3 | 3.7  ± 1.0 | 4.8  ± 1.7 | 8.5  ± 2.7 |
| MetRS                 | 50.9 ± 9.1 | 110.8 ± 10.8 | 43.4 ± 13.7 | 60.6 ± 25.1 |
| MetRS and MTF         | 168.8 ± 5.3 | 120.1 ± 5.5 | 166 ± 38 | 62.1 ± 9.1 |

a The CAT activities are normalized to that of tRNA<sub>16S</sub> (CUA), which is set as 100%
b The data are the same as in Table I, used here for comparison.
c Average of three separate assays on three individual extracts. All the other numbers are average of two to six individual assays.

This strain has a tendency to lose the pRSV<sub>p</sub>CATam1.2.5 plasmid carrying the CAT reporter and the tRNA gene at a high frequency. Only a fraction of the cells from an overnight culture grown in the presence of ampicillin and tetracycline still contain the pRSV plasmid. Because the cell density of the fresh culture from an inoculum of the overnight culture was less than that of the other cultures at the time of harvesting, two to two and a half times as much of the fresh culture of this strain was used for preparation of the cell extracts compared to the other cultures (see "Materials and Methods").

H. J. Drabkin and U. L. Rajbhandary, unpublished data.
tRNA, which migrates between fLys-tRNA and fMet-tRNA (see Fig. 4 of MetRS and MTF. CA274 transformants overproducing MTF, MetRS, or both invivo previous conclusions based on small effect lowering the tRNA context. Mutation of G4:C69 base pair to C:G base pair in tRNA of the G4:C69 base pair on formylation depends on the results show that protein synthesis in formylation of tRNA in initiation of protein synthesis, these confirming previous conclusions (5, 6) on the importance of formylation of tRNA in initiation of protein synthesis, these results show that protein synthesis in E. coli can be initiated with formyllysine. Thus, besides methionine, the list of amino acids shown to initiate protein synthesis in E. coli now includes Lys in addition to Gin, Ile, Phe, Val, Cys, Trp, and Tyr (5, 14, 27–29).

The clear effect in vivo of the presence of the G4:C69 base pair in formylation of the Mi:2/4 and Mi:2/4,5 tRNAs, derived from E. coli elongator methionine tRNA, is in agreement with previous conclusions based on in vitro experiments that importance of the G4:C69 base pair on formylation depends on the tRNA context. Mutation of G4:C69 base pair to C:G base pair in the E. coli initiator tRNA sequence background has a relatively small effect lowering $V_{\text{max}}/K_m$ in formylation by a factor of about 4.7 (9). In contrast, mutation of G4:C69 in an elongator methionine tRNA background has a much more severe effect, lowering $V_{\text{max}}/K_m$ by a factor of about 100 (10).

It is important to note that in spite of the observed formylation of the Mi:2/4 and Mi:2/4,5 tRNAs, these tRNAs are still very poor substrates for formylation when aminoacylated with lysine. Formylation of these tRNAs is partial and detectable only in cells overproducing MetRS and MTF. In contrast, essentially all of the tRNAs aminoacylated with methionine is formylated in cells overproducing MetRS and MTF. These results further highlight the important role of the amino acid discussed above in formylation.

Recognition of tRNAs by LysRS—in attempts to generate amber suppressors derived from different tRNAs, Abelson, Miller and colleagues (30) found that these suppressors could be divided into three classes: those that inserted the predicted amino acid, those that inserted predominantly glutamine, and those that inserted predominantly lysine. It is not known why amber suppressors derived from some tRNAs such as the E. coli initiator tRNA or tRNA$^{Met}$ are aminoacylated with glutamine, whereas those derived from the elongator methionine tRNA and tRNA$^{me}$ are aminoacylated with lysine. One possible correlation, based on genetic and biochemical studies of tRNA mutants (22, 31–34) and x-ray crystallographic analysis of the E. coli glutaminyl-tRNA synthetase-tRNA$^{Gln}$ complex (35), is that tRNAs aminoacylated with glutaminyl-tRNA synthetase either have a weak base pair between nucleotides 1 and 72 that can be easily disrupted by the enzyme or have G73 in the discriminator position. G73 stabilizes a form of the acceptor stem structure in which the 1:72 base pair is disrupted and the tRNA-CCA end is curled back toward the anticodon. However, an anticodon mutant of E. coli asparagine tRNA that has a weak U1:A72 base pair and G73 is aminoacylated with lysine and not with glutamine (36). Also, although the aminoacylation of the amber suppressor derived from the elongator methionine tRNA, which has a strong G1:C72 base pair, with lysine rather than glutamine could be rationalized on this basis (30), the current finding that the Mi:2 tRNA with a C1x:A72 mismatch is also aminoacylated with lysine is not easily explained. It is possible that the Mi:2 tRNA and tRNA$^{Asn}$ contains other determinants for LysRS that predominate over the above features. Alternatively, they have features that act as negative determinants toward E. coli glutaminyl-tRNA synthetase (37). The finding that the Mi:2 tRNA with a C1x:A72 mismatch is aminoacylated with lysine in vivo suggests that the presence of a G1:C72 base pair found in tRNA$^{Lys}$ is not essential for its aminoacylation by LysRS.

The Mi:2/4,5 tRNA is also aminoacylated with lysine in vivo. This tRNA has the first 5 base pairs in the acceptor stem, the last 3 base pairs in the anticodon stem, and the entire sequence of the anticodon loop identical to the U35A36 mutant of E. coli initiator tRNA. The latter tRNA is aminoacylated with glutamine in vivo and in vitro (14, 22, 33). These results suggest that the sequence and/or structural features in the Mi:2/4,5 tRNA that determine whether the tRNA is aminoacylated in vivo with glutamine or lysine probably reside outside of these regions.

It is interesting that changing the fourth and in particular the fifth base pair in the acceptor stem of the Mi:2 tRNA from U4:A69 and A5:U68 to G:C base pairs makes these tRNAs better substrates for LysRS in vivo. The lysine tRNA of E. coli does not have G:C base pairs at these positions, in fact it has U4:A69 and C5:G68 (38). It is possible that these changes in the Mi:2 tRNA affect the acceptor stem structure in such a way that it fits better into the catalytic pocket of LysRS. It is also possible that LysRS interacts directly with the fifth base pair of lysine tRNA. The G5:C68 base pair of the Mi:2/5 tRNA could provide some of the same contacts as that provided by a C5:G68 base pair (39). In this regard, it may be relevant that an amber suppressor derived from E. coli tRNA$^{Arg}$ with a C5:G68 base pair is partially aminoacylated in vivo with lysine, whereas that derived from another species of tRNA$^{Arg}$ with a U5:A68 base pair is instead partially aminoacylated with glutamine (40, 41).

Finally, whether a mutant tRNA is aminoacylated in vivo with lysine, glutamine, methionine, or other amino acids depends upon competition in cells between the various amino-
they are for either glutaminyl-tRNA synthetase or for MetRS. Because the lysine side chain has a positive charge, a tRNA aminoacylated with glutamine, methionine, and most other amino acids by its slower mobility on polyacrylamide gels run under acidic conditions (13, 19) (lanes 3 and 4 of Fig. 6, A–D; see also Fig. 4 of Ref. 13). Inspection of autoradiograms corresponding to Fig. 5 exposed for long periods shows that none of the mutant tRNAs are aminoacylated with any amino acid other than lysine. Furthermore, whereas overproduction of LysRS leads to essentially complete aminoacylation of each of the mutant tRNAs with lysine (lane 3 of Fig. 5, A–D), overproduction of glutaminyl-tRNA synthetase (data not shown, see Fig. 4 of Ref. 13) or of MetRS (lane 3 of Fig. 6, A–D and Fig. 4 of Ref. 13) still leaves most of the tRNA in the uncharged form. These data indicate that Mi:2, Mi:2/4, Mi:2/5, and Mi:2/4,5 tRNAs are all better substrates for LysRS than for MetRS.

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REFERENCES

1. Kozak, M. (1983) Microbiol. Rev. 47, 1–45
2. Gold, L. (1988) Annu. Rev. Biochem. 57, 199–233
3. Gualerzi, C. O., and Pon, C. L. (1990) Biochemistry 29, 5881–5889
4. Marcker, K., and Sanger, F. (1964) J. Mol. Biol. 8, 835–840
5. Varshney, U., Lee, C.-P., Mandal, N., Varshney, U., Brahman, B., and RajBhandary, U. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9262–9266
6. Guillon, J.-M., Seong, B. L., and RajBhandary, U. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 334–338
7. Varshney, U., Lee, C.-P., and RajBhandary, U. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2305–2309
8. Varshney, U., and RajBhandary, U. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1586–1590
9. Chow, C. M., and RajBhandary, U. L. (1993) J. Biol. Chem. 268, 12855–12863
10. Marangoz, D., and RajBhandary, U. L. (1995) J. Biol. Chem. 270, 12203–12209
11. Wessel, D., and Flugge, U. I. (1984) Anal. Biochem. 136, 141–143
12. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
13. Varshney, U., and RajBhandary, U. L. (1991) J. Biol. Chem. 266, 24712–24718
14. Guillon, J.-M., Meinhel, Y., Blanquet, S., and Fayat, G. (1993) J. Bacteriol. 175, 4507–4514
15. Varshney, U., and RajBhandary, U. L. (1992) J. Bacteriol. 174, 7819–7826
16. Seong, B. L., Lee, C.-P., and RajBhandary, U. L. (1989) J. Biol. Chem. 264, 6504–6508
17. Giege, R., Ebé, J. P., and Clark, B. F. C. (1973) FEBS Lett. 30, 1–295
18. Wakao, H., Ronley, P., Westhof, E., Laalami, S., Grunberg-Manago, M., Ebé, J.-P., Ehresmann, C., and Ehresmann, B. (1989) J. Biol. Chem. 264, 20363–20371
19. Sundari, R., Stringer, E. A., Schulman, L. H., and Maitra, U. (1976) J. Biol. Chem. 251, 3338–3345
20. Wigner, T., Gross, M., and Sigler, P. B. (1984) J. Biol. Chem. 259, 4706–4709
21. Chattapadhyay, R., Pelka, H., and Schulman, L. H. (1990) Biochemistry 29, 4263–4268
22. Pallandik, L., and Schulman, L. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3872–3876
23. Pallandik, L., Li, S., and Schulman, L. H. (1992) J. Biol. Chem. 267, 7221–7223
24. Wignall, J., Kleina, L. G., Masson, J.-M., Abelson, J., and Miller, J. H. (1990) J. Mol. Biol. 213, 719–726
25. Smith, J. D., and Celis, J. E. (1973) Nat. New Biol. 243, 66–71
26. Shimura, Y., Aono, H., Ozeki, H., Sarabhai, A., Lamfrom, H., and Abelson, J. (1972) FEBS Lett. 22, 144–149
27. Schulman, L. H., and Pelka, H. (1985) Biochemistry 24, 7309–7314
28. Dyson, M. R., Mandal, N., and RajBhandary, U. L. (1993) Biochimie (Paris) 75, 1051–1060
29. Roil, M. A., Perona, J., Soll, D., and Steitz, T. A. (1989) Science 246, 1133–1142
30. Li, S., Pelka, H., and Schulman, L. H. (1993) J. Biol. Chem. 268, 18335–18339
31. Weggand-Gurasevic, L., Schwob, E., and Söll, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2010–2014
32. Sprinzl, M., Hartman, T., Weber, J., Blank, J., and Zeidler, R. (1989) Nucleic Acids Res. 17, r1–r172
33. Seeman, N. C., Rosenberg, J. M., and Rich, A. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 804–808
34. McClain, W. H., and Foss, K. (1988) Science 241, 1804–1807
35. Yarus, M. (1972) Nat. New Biol. 239, 106–108
36. Swanson, R., Høbern, P., Sumner-Smith, M., Uemura, H., Watson, L., and Söll, D. (1988) Science 242, 1548–1551
37. Hou, Y.-M., and Schimmel, P. (1989) Biochemistry 28, 4942–4947
38. Meinhel, T., Guillon, J.-M., Meinhel, Y., and Blanquet, S. (1993) J. Bacteriol. 175, 993–1000
39. Kumar, N. V., and Varshney, U. (1994) Curr. Sci. (Bangalore) 67, 728–734