Suppressor Mutations in *Escherichia coli* Methionyl-tRNA Formyltransferase That Compensate for the Formylation Defect of a Mutant tRNA Aminoacylated with Lysine†

Yan Li,‡ Vaidyanathan Ramesh,‡ Dev Mangroo, Charu Taneja, and Uttam L. RajBhandary*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received March 17, 2000; Revised Manuscript Received April 28, 2000

ABSTRACT: The specific formylation of initiator methionyl-tRNA by methionyl-tRNA formyltransferase (MTF) is important for the initiation of protein synthesis in eubacteria such as *Escherichia coli*. In addition to the determinants for formylation present in the initiator tRNA, the nature of the amino acid attached to the tRNA is also important for formylation. We showed previously that a mutant tRNA aminoacylated with lysine was an extremely poor substrate for formylation. As a consequence, it was essentially inactive in initiation of protein synthesis in *E. coli*. In contrast, the same tRNA, when aminoacylated with methionine, was a good substrate for formylation and was, consequently, quite active in initiation. Here, we report on the isolation of suppressor mutations in MTF which compensate for the formylation defect of the mutant tRNA aminoacylated with lysine. The suppressor mutant has glycine 178 changed to glutamic acid. Mutants with glycine 178 of MTF changed to aspartic acid, lysine, and leucine were generated and were found to be progressively weaker suppressors. Studies on allele specificity of suppression using different mutant tRNAs as substrates suggest that the Gly178 to Glu mutation compensates for the nature of the amino acid attached to the tRNA. We discuss these results in the framework of the crystal structure of the MTF·fMet-tRNA complex published recently.

Two species of methionine tRNA are found in all organisms studied to date (*1*). One of these, the initiator, is used specifically for the initiation of protein synthesis whereas the other, the elongator, is used to insert methionine internally into a polypeptide chain (*2*−*4*). In eubacteria, mitochondria, and chloroplasts, the initiator tRNA is used as formylmethionyl-tRNA (fMet-tRNA).[1] The formylation reaction, catalyzed by methionyl-tRNA (Met-tRNA) formyltransferase (MTF), is important for the initiation of protein synthesis in eubacteria such as *E. coli* and is highly specific (*5*). The enzyme formylates the initiator tRNA but no other tRNA including the elongator Met-tRNA (*6*). The most important determinants in the tRNA used by MTF to distinguish the initiator tRNA from other tRNAs are clustered in the acceptor stem (*7*−*10*). Besides these determinants, the nature of the amino acid attached to the tRNA also has a significant effect on formylation (*11*−*14*).

For functional studies of mutant initiator tRNAs in *E. coli*, we previously reported on a strategy based on the use of mutant tRNAs carrying an anticodon sequence change from CAU→CUA (*15*). The CUA anticodon sequence allows the assessment of the activity of mutant tRNAs in initiation in *E. coli* by measuring the levels of chloramphenicol acetyltransferase (CAT) activity from a reporter CAT gene carrying UAG as the initiation codon. Using this strategy, we identified the features in the initiator tRNA critical for its activity in initiation. These features included the determinants in the acceptor stem important for formylation and determinants in the anticodon stem important for directing the initiator fMet-tRNA to the P site on the ribosome (*5*, *16*, *17*). However, transplanting these features into an elongator methionine tRNA did not, in itself, produce a tRNA that was active in initiation. The mutant elongator tRNA was active in initiation only in cells overproducing the *E. coli* methionyl-tRNA synthetase (*12*, *18*). The reason for it was that the mutant tRNA was not formylated when it was aminoacylated with lysine but was formylated when it was aminoacylated with methionine (*13*, *14*).

The strict requirement for formylation of aminoacyl-tRNAs for their activity in initiation combined with the strategy developed for functional studies of mutant tRNA provides an approach for the isolation of suppressor mutations in MTF (*19*). Here, we describe the isolation of a suppressor mutation in MTF which allows the enzyme to formylate the mutant elongator methionine tRNA that is aminoacylated with lysine and, thereby, converts it to a tRNA that is active in initiation in vivo. The mutant elongator methionine tRNA, designated Mi:2/5 (Figure 1), has mutations in the acceptor stem necessary for formylation of tRNAs by MTF, mutations in the anticodon stem necessary for directing the tRNA to the ribosomal P site, and mutations in the anticodon sequence necessary for it to read the UAG present as an initiation

† This work was supported by Grant R37GM17151 from the National Institutes of Health.

* Corresponding author. Tel.: (617) 253-4702, FAX: (617) 252-1556, e-mail: bhandary@mit.edu.

‡ The first two authors contributed equally to this work.

[1] Abbreviations: MTF, methionyl-tRNA formyltransferase; CAT, chloramphenicol acetyltransferase; LysRS, lysyl-tRNA synthetase; Met-tRNA, methionyl-tRNA; fMet-tRNA, formylmethionyl-tRNA; Lys-tRNA, lysyl-tRNA; fLys-tRNA, formyllysyl-tRNA; $tRNA_2^{fMet}$, formylmethionine tRNA species 2 of *E. coli*; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

10.1021/bi000625+ CCC: $19.00
Published on Web 06/10/2000

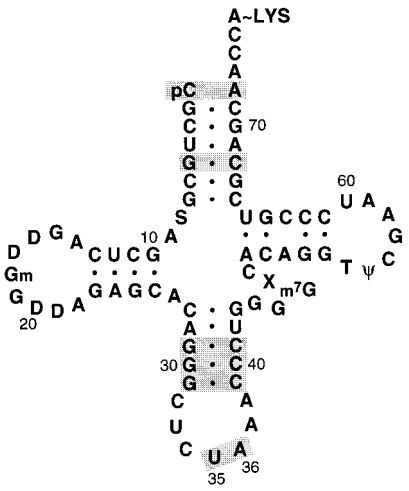

FIGURE 1: Cloverleaf structure of the Mi:2/5 mutant of *E. coli* elongator methionine tRNA used for isolation of suppressor mutations in MTF. Shaded areas indicate the sites of mutation in the elongator tRNA. This tRNA is aminoacylated with lysine in *E. coli*.

codon in the reporter *CATam1.2.5* gene (*13*). The suppressor mutation in MTF has a glycine to glutamic acid change at amino acid 178. We show that the mutant tRNA is formylated in cells expressing the G178E mutant MTF but not in cells expressing the wild-type MTF. Comparison of the kinetic parameters of the mutant enzyme and the wild-type enzyme using the wild-type initiator Met-tRNA as substrate shows that suppression in vivo is not due to a nonspecific increase in the catalytic rate of the mutant enzyme. Comparison of the suppressor activity of the G178E mutant with three other mutant MTFs (G178D, G178K, and G178L) generated by site-specific mutagenesis showed that the G178E mutant was by far the best suppressor. Studies on allele specificity of suppression using other related mutant tRNAs suggest that the G178E suppressor mutation compensates for the nature of the amino acid attached to the tRNA. We discuss these results in the framework of the recently determined crystal structure of the *E. coli* MTF·fMet-tRNA complex (*20*).

MATERIALS AND METHODS

Strains and Plasmids. E. coli strains CA274 *HfrH lacZ125am trpEam*, JM109 (*21*), DH5α, and Novablue (DE3) pLysS (obtained from Novagen) were used as hosts in this study. The pGFIBI-MTF vector was described previously (*22*); pET3d was purchased from Novagen. Plasmid pRSVpCATam1.2.5 Mi:2/5 LysRS (Amp^R) contains the Mi:2/5 mutant elongator methionine tRNA gene (Figure 1), the reporter *CATam1.2.5* gene, and the lysyl-tRNA synthetase (LysRS) gene. Other variants of this plasmid contain the Mi:2/4/5 or the Mi:3/A51:U63 mutant elongator tRNA genes instead of the Mi:2/5 tRNA gene. The LysRS gene was excised as an ~2 kb *Sac*I fragment from pAC1LysRS (*23*); the sticky ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I and cloned into the *Hpa*I site of the pRSVpCATam1.2.5 plasmid carrying the Mi:2/5 mutant tRNA gene. Alternatively, the LysRS gene was excised as an ~2 kb *Ecl*136II blunt-ended fragment and cloned into the *Hpa*I site of the pRSVpCATam1.2.5 plasmid carrying the Mi:2/4/5 or the Mi:3/A51:U63 mutant elongator methionine tRNA genes. The orientations of the LysRS gene in all these constructs are the same and are indicated in Figure 2. The plasmid pACDFMT (Tet^R) contains the *fmt* gene for *E. coli* MTF (*19*).

Subcloning of the Wild-Type and Mutant MTF Gene Fragment into pACD FMS-FMT. The plasmid pACD FMS-FMT contains the *fms-fmt* genes for *E. coli* peptide deformylase and MTF, respectively. In this construct, the *fmt* gene is part of a dicistronic operon under control of the *fms* promoter (*24*). A 280 bp *Xcm*I−*Msc*I fragment of the *fmt* gene, including within it the sites of the various G178 mutations, was subcloned into the *Xcm*I−*Msc*I site of the pACD FMS-FMT vector.

Medium. Medium used to cultivate *E. coli* was LB (1% tryptone, 0.5% yeast extract, 1% NaCl). When needed, ampicillin and tetracycline were added to a final concentration of 100 μg/mL and 15 μg/mL, respectively.

tRNA. Total *E. coli* tRNA was isolated by phenol extraction. The initiator $tRNA_2^{fMet}$ species was purified by gel electrophoresis of total tRNA isolated from a transformant overproducing this tRNA (*25*).

Synthesis of tRNA-Sepharose Matrix. (A) Preparation of Hydrazide-Sepharose. The tRNA-Sepharose column was prepared as described (*26*). Cyanogen bromide activated Sepharose (15 g) was washed with 3 L of 1 mM HCl and resuspended in 0.2 M $NaHCO_3$, pH 10.0. Hydrazine (2

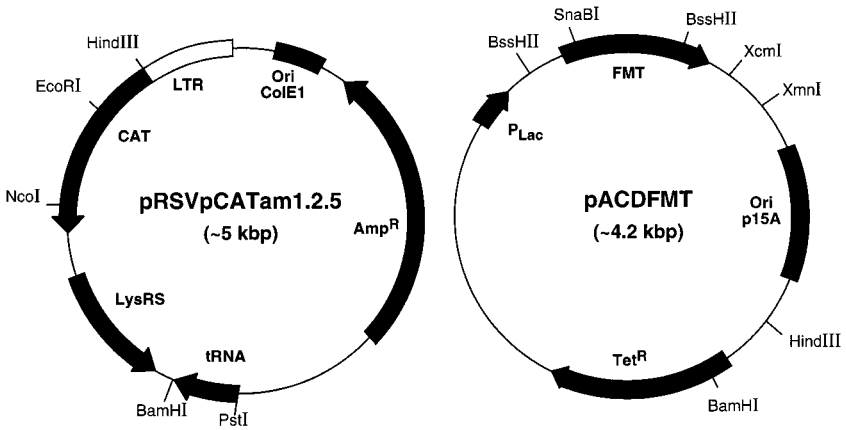

FIGURE 2: Plasmids used for isolation of suppressor mutations in MTF. pRSVp*CATam1.2.5* (left) contains the gene for the *CATam1.2.5* reporter, the Mi:2/5 mutant tRNA gene, and the gene for *E. coli* LysRS. The pACDFMT plasmid (right) contains the *FMT* gene for *E. coli* MTF and was used for the isolation of mutations in the *FMT* gene by using MNNG mutagenesis (details in text). LTR, long terminal repeat.

mmol/mL of gel) in water was adjusted to pH 10.0 with NaOH, and $NaHCO_3$ was added to a final concentration of 0.2 M. The solution of hydrazine was added to the gel suspension, and the reaction was allowed to proceed for 5 h at room temperature with shaking. The hydrazide-Sepharose was washed with 2 L of 0.2 M $NaHCO_3$ and 1 L of 0.1 M sodium acetate, pH 5.0, buffer containing 10 mM $MgCl_2$.

(B) Periodate Oxidation of tRNA. Total *E. coli* tRNA (930 A_{260} units) was dissolved in 0.2 M sodium acetate, pH 5.0, buffer containing 20 mM $MgCl_2$ at a final concentration of 8 mg/mL. An equal volume of 20 mM sodium periodate in water was added, and the oxidation reaction was allowed to proceed for 1 h at room temperature. Excess sodium periodate was precipitated with 0.2 M KCl at 4 °C for 10 min. The oxidized tRNA was precipitated with 2.5 volumes of ethanol at 4 °C, dissolved in 0.1 M sodium acetate, pH 7.5, 10 mM $MgCl_2$, and dialyzed against the same buffer at 4 °C.

(C) Reaction of Periodate-Oxidized tRNA with Hydrazide-Sepharose. The solution of oxidized tRNA was added to the hydrazide-Sepharose suspended in 0.1 M sodium acetate, pH 5.0, 10 mM $MgCl_2$ and incubated overnight at room temperature with shaking. The gel was washed with 1 L of 1 M NaCl and resuspended in 0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$. Sodium borohydride (3 mg/mg of tRNA) was added, and after 2 h of incubation at room temperature with shaking, the gel was washed extensively with 20 mM imidazole hydrochloride, pH 7.6, 10 mM β-mercaptoethanol (β-MSH), and 100 mM KCl. The capacity of the column was determined to be 0.5 mg of tRNA/mL of gel.

Overproduction and Purification of Wild-Type MTF. A *Pvu*II−*Afl*III fragment containing the coding region of the wild-type *fmt* gene was obtained from the pGFIBI-MTF vector and cloned into the *Bam*HI and *Nco*I sites in the pET3d expression vector. Incompatible cohesive end in the vector (*Bam*HI) was filled in using the Klenow fragment of DNA polymerase I in the presence of dNTPs. *E. coli* Novablue harboring the pET vector carrying the *fmt* gene was grown overnight at 37 °C in 3 mL of LB medium containing 100 μg/mL ampicillin, 7.5 μg/mL tetracycline, and 30 μg/mL chloramphenicol. An aliquot of the overnight culture was diluted 500-fold into 100 mL of LB medium containing antibiotics and grown overnight at 37 °C. The overnight culture was diluted 50-fold in 1 L of fresh medium containing antibiotics and grown at 37 °C to an A_{600} of 0.6. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for 2 h. The cells were pelleted by centrifugation. The cell pellet (16 g wet weight) was resuspended in 63 mL of 25 mM Tris-HCl, pH 8.0, buffer containing 2 mM β-MSH and 1 mM EDTA, and lysozyme (0.5 mL of 8 mg/mL) was added. After 15 min at room temperature, a solution (1.6 mL) of DNase I (1 mg/mL in 0.1 M $MgCl_2$) was added to the cell lysate. After 30 min at room temperature, the cell lysate was centrifuged for 1 h at 100000*g* at 4 °C, and the supernatant was used for purification of MTF. All subsequent steps were performed at 4 °C.

MTF was purified to homogeneity using a modification of the procedure involving chromatography on DEAE-Sepharose, hydroxyapatite, and tRNA-Sepharose (*27*). Fractions containing the purified enzyme from the final tRNA-Sepharose step were pooled, dialyzed against 20 mM imidazole hydrochloride, pH 7.6, buffer containing 10 mM β-MSH, 150 mM KCl, and 50% glycerol (w/v), and stored at −20 °C. Determination of the specific activity of MTF at each step indicated that the procedure resulted in an overall 14-fold purification of the enzyme. The recovery of the enzyme was 69%, and the yield of pure MTF from 2 L of culture was 57 mg.

Generation of Antibody against MTF. MTF (175 μg in 300 μL of buffer) was mixed with an equal volume of complete Freund adjuvant and injected into a rabbit. After 4 weeks, the rabbit was inoculated with another 175 μg of MTF in complete Freund adjuvant. Another inoculum was given 4 weeks later. The serum was isolated and stored at −20 °C.

Mutagenesis and Selection of Suppressor Mutations in MTF. The mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was used for introducing mutations into the *fmt* gene (*19*, *28*). The *E. coli* DH5α cells harboring the plasmid pACDFMT were treated with MNNG at a final concentration of 50 μg/mL. Plasmid DNA containing a pool of mutant pACDFMT was isolated as described previously (*19*). This DNA was used to transform the recipient strain CA274 containing pRSVp*CATam1.2.5* with the Mi:2/5 tRNA gene and the LysRS gene. The cells were allowed to grow for 4 h and then plated on ATC_{100} plates (LB containing 100 μg/mL ampicillin, 15 μg/mL tetracycline, and 100 μg/mL chloramphenicol). More than 100 chloramphenicol-resistant (Cam^R) transformants were obtained after 18−24 h of incubation at 37 °C. Eighty-five of these Cam^R colonies were streaked on ATC_{100} plates to confirm the phenotype. Of the 85 colonies, 20 grew again on the ATC_{100} plates. Plasmid DNAs isolated from 12 of the 20 Cam^R colonies were used to transform CA274 for a second time. Tetracycline resistance was used to select for transformants carrying the pACD plasmid with the *fmt* gene. The tetracycline-resistant transformants were tested for ampicillin-sensitivity to make sure that the pRSVp*CATam1.2.5* plasmid was not included among the transformants. The *fmt*-containing plasmids from the 12 Cam^R mutants were further screened by subcloning of an ≈1 kbp *Bss*HII fragment containing the *fmt* gene (including 100 bp upstream but lacking the sequence for the C-terminal 20 amino acids) from these plasmids into a fresh pACDFMT vector. Plasmid DNAs isolated were then used to transform CA274 cells harboring the untreated pRSV vector carrying the Mi:2/5 tRNA and LysRS genes. Transformants were streaked out on ATC_{100} plates to select for Cam^R colonies. Of the 12 plasmids, 2 yielded transformants which were Cam^R. The plasmid DNAs from the Cam^R colonies were isolated and used for sequence analysis of the *fmt* gene.

Preparation of E. coli Cell Extract. The overnight culture was diluted 20-fold in fresh media and grown at 37 °C for 3−4 h. The cells were harvested from 1.2 mL of culture and lysed as described before (*5*). Protein concentration was determined by the Bio-Rad dye binding assay using IgG as the standard (*29*).

Assays for CAT, β-Lactamase, and MTF. CAT and β-lactamase activities in crude cell lysate were assayed as described (*5*). To minimize the effect of any changes in the copy numbers of the pRSVpCATam1.2.5 plasmid, the specific activities of CAT were normalized to the specific activities of β-lactamase in the same cell extract. Assay for MTF used a two step reaction, aminoacylation followed by formylation as described before (*30*).

Immunoblot Analyses. A 5−10 μg sample of protein extract was separated on a 12% SDS−polyacrylamide gel and transferred onto an Immobilon-P membrane (Millipore). After transfer, membranes were blocked in 5% nonfat dry milk in TBS (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.05% Tween-20) at room temperature overnight. Membranes were then washed 3 times for 10 min each with TBS and incubated with antibody against either MTF (15 000-fold diluted) or CAT (10 000-fold diluted) and β-lactamase (20 000-fold diluted) for 1 h at room temperature. The membranes were subsequently washed 3 times for 10 min each and incubated with peroxidase-conjugated donkey anti-rabbit antibody (10 000-fold diluted) for 1 h. After washing 3 times for 10 min each, the bound antibodies were detected with a 1:1 mixture of the two enhanced chemiluminescence reagents supplied with the Amersham kit.

Isolation and Northern Blot Analysis of tRNAs. The procedure used was as described before (*31*) except that the 7% acid−urea−polyacrylamide gel was made and run in 0.2 M NaOAc, pH 5, running buffer instead of 0.1 M NaOAc. 0.02 A_{260} unit of tRNA was loaded, and the probe was a 5′-^{32}P-labeled deoxyribooligonucleotide complementary to nucleotides 25−45 of the Mi:2/5 tRNA. Deacylation of aminoacyl-tRNA was carried out by incubating the tRNA with 0.1 M Tris, pH 9.4, at 37 °C for 1 h.

RESULTS

Isolation of Suppressor Mutations in MTF. The strategy for the isolation of suppressor mutations in MTF consists of the use of the *CATam1.2.5* gene carrying the UAG initiation codon as a reporter in cells carrying a mutant tRNA, which has the potential to read the UAG initiation codon but is inactive in initiation because it is defective in formylation (Figure 2, left). Cells carrying the plasmid with the *CATam1.2.5* gene and the mutant tRNA gene do not make CAT and are sensitive to chloramphenicol. Suppressor mutations in MTF, which allow the enzyme to formylate the mutant tRNA, would allow the synthesis of CAT and would, thereby, confer chloramphenicol resistance phenotype on the cells (*19*). The gene for MTF is carried on a separate plasmid (Figure 2, right). This is in addition to the chromosomal copy of the MTF gene.

The mutant tRNA used for this purpose, designated previously as Mi:2/5 tRNA, is derived from the *E. coli* elongator methionine tRNA (Figure 1). It carries the determinants in the acceptor stem necessary for it to be a substrate for MTF (the C1×A72 mismatch and the G2·C71 and C3·G70 base pairs), the determinants in the anticodon stem necessary for it to bind to the ribosomal P site (G29G30G31·C39C40C41 base pairs), and the CAU→CUA anticodon sequence mutation necessary for it to read the UAG initiation codon. In addition, the tRNA has a G5·C68 base pair mutation in the acceptor stem, which makes it a slightly better substrate for formylation (*13*). The sites of these mutations are highlighted in Figure 1. Because of the anticodon sequence change, this mutant tRNA is aminoacylated with lysine (*32*). Although it contains the most important determinants for formylation, it is an extremely poor substrate for formylation because it is aminoacylated with lysine (*12*, *13*). As a consequence, the Mi:2/5 tRNA is essentially inactive in initiation even in cells overproducing MTF (*13*).

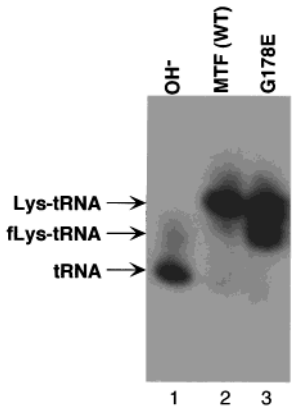

FIGURE 3: Northern blot analysis of the Mi:2/5 mutant tRNA isolated from *E. coli* CA274 transformants overproducing LysRS and either wild-type MTF (lane 2) or the G178E mutant (lane 3). Lane 1 provides a marker of deacylated tRNA obtained by mild alkali treatment of total tRNA from the same preparation as that used in lane 2.

To ensure that the Mi:2/5 tRNA is maximally aminoacylated with lysine in vivo, *E. coli* LysRS gene was also included in the pRSVpCATam1.2.5 plasmid for overproduction of LysRS (Figure 2, left).

The mutagenesis of DH5α cells carrying the gene for MTF in the pACDFMT plasmid (Figure 2, right) with MNNG and isolation of the plasmid DNA from the mutagenized culture have been described before (*19*). The pool of mutagenized plasmid DNA was used to transform *E. coli* CA274 carrying the pRSVpCATam1.2.5 plasmid (Figure 2, left). Transformants were selected for growth on plates containing ampicillin, tetracycline, and 100 μg/mL chloramphenicol (ATC_{100}). Of the 85 chloramphenicol-resistant colonies picked for analysis, 20 grew for a second time on the ATC_{100} plates. Purification of the pACDFMT plasmid from 12 of these cells followed by subcloning of the MTF gene into a fresh pACD vector and transformation of *E. coli* CA274 carrying the pRSVpCATam1.2.5 plasmid showed that 2 of them conferred chloramphenicol resistance on the transformants. The MTF genes in the pACDFMT plasmids from the two CAM^R transformants were sequenced. Both contained a GGG→GAG mutation resulting in a Gly178 to Glu change in MTF; one of them also contained a GCT→ACT mutation resulting in an Ala55 to Thr change. The G·C→A·T transition mutations in the MTF gene are consistent with the known preference of MNNG-induced mutations (*28*).

A single mutant with Ala55 to Thr change was generated by site-specific mutagenesis and tested for its activity as a suppressor in vivo. In contrast to the G178E mutation, the A55T mutation did not suppress the formylation defect of the Mi:2/5 tRNA. Therefore, the G178E mutation in MTF compensates for the formylation defect of the Mi:2/5 mutant tRNA in both of the suppressor mutants isolated.

Analysis for Formylation of Mi:2/5 tRNA in Cells Overproducing the Wild-Type MTF or the G178E Mutant. The chloramphenicol resistance phenotype of *E. coli* CA274 carrying the pRSVpCATam1.2.5 and the G178E pACDFMT plasmid is due to formylation of the Mi:2/5 tRNA, aminoacylated with lysine, by the G178E mutant MTF. Figure 3 shows the results of acid−urea gel electrophoresis to separate the various forms of the Mi:2/5 tRNA isolated from cells overproducing the wild-type MTF or the G178E mutant,

Table 1: Kinetic Parameters in Formylation of Wild-Type *E. coli* Initiator tRNA Using Wild-Type and the G178E Mutant MTF

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	relative k_{cat}/K_m
wt	0.51 ± 0.02	13.9 ± 6.2	27.3	1
G178E	0.72 ± 0.21	8.9 ± 1.1	12.4	0.45

followed by Northern blot analysis using a probe complementary to nucleotides 25−45 of the Mi:2/5 tRNA (*31*). In cells overproducing the wild-type MTF, the tRNA is essentially quantitatively aminoacylated, but there is little or no formylation of the Lys-tRNA as indicated by the absence of a band corresponding to fLys-tRNA (lane 2). In contrast, in cells overproducing the G178E mutant MTF, there is a strong band corresponding to fLys-tRNA (lane 3). Approximately, 50% of the Mi:2/5 tRNA is present as fLys-tRNA.

Comparison of Catalytic Properties of Wild-Type MTF and the G178E Mutant Using $tRNA_2^{fMet}$ as Substrate. The formylation of the Mi:2/5 tRNA in vivo by the G178E mutant MTF could be due to a general nonspecific increase in the catalytic activity of the mutant enzyme compared to the wild-type enzyme. To test this possibility, the His-tagged wild-type and mutant enzymes were purified by affinity chromatography (*19*, *33*) and used to study the kinetic parameters in formylation of the wild-type *E. coli* initiator methionyl-tRNA (Met-tRNA). The data in Table 1 show that the k_{cat}/K_m for the mutant enzyme is, if anything, slightly lower than that for the wild-type enzyme.

Generation of Gly178 to Leu, Asp, and Lys Mutations by Site-Specific Mutagenesis and Analysis of Their Activity in Suppression in Vivo. To investigate how the G178E mutant MTF suppresses the formylation defect of the Mi:2/5 tRNA, Gly178 was changed to Leu, Asp, and Lys by site-specific mutagenesis of the MTF gene. Transformants carrying the G178L mutant were easily isolated, but the transformants expressing the G178D and G178K mutants proved difficult to work with because cells carrying these mutants gave varying levels of expression of MTF, indicating instability of the plasmid or of the expressed MTF mutants. The pACDFMT plasmid expresses MTF from a lac promoter, a phage T5 gene 10 Shine−Dalgarno sequence, and has AUG as the initiation codon. In contrast, the chromosomal gene for MTF, which is part of a FMS-FMT dicistronic operon, expresses MTF from a weak promoter and has GUG instead of AUG as the initiation codon (*24*). Partly because of these differences, cells carrying the pACDFMT plasmid overproduce MTF by more than 200-fold over that from the chromosomal locus. In view of the possible instability of the pACDFMT plasmid carrying the G178D and G178K mutations, the wild type and all of the mutant MTF genes were subcloned into the pACD vector carrying the *E. coli* FMS-FMT locus (*22*). The extent of overproduction of MTF from this vector was about 22−23-fold over that from the chromosomal locus (data not shown).

The mutant MTF genes were introduced into *E. coli* CA274 carrying the pRSVpCATam1.2.5 plasmid, and transformants were screened for growth on plates containing ampicillin, tetracycline, and chloramphenicol at 25, 50, and 75 μg/mL. All transformants grew on plates containing 25 and 50 μg/mL chloramphenicol, whereas only transformants carrying the G178E mutant showed clear growth at 75 μg/mL after 18 h of incubation. Thus, compared to the G178E mutant, the G178L, G178D, and G178K mutants are weaker suppressors.

CAT Protein Levels and Activity in Extracts of Cells Carrying the pRSVpCATam1.2.5 Plasmid and the Various Mutant MTF Genes. Assays of CAT protein levels and CAT activity in cell extracts were next used to compare the suppressor activity of the various mutant MTF genes. Figure 4A shows the results of an immunoblot analysis of extracts from cells carrying either the empty pACD vector or the various MTF genes, using anti-CAT and anti-β-lactamase antibodies. A band corresponding to the CAT protein is clearly seen in extracts of cells expressing the G178E mutant MTF gene (lane 3). This band is absent in extracts of cells carrying the empty vector or the wild-type MTF gene (lanes 1 and 2) and is much weaker or absent in extracts of cells carrying the other mutant MTF genes (lanes 4−6). The β-lactamase levels are approximately the same in all of the extracts.

As a control, the extracts were also probed with a polyclonal rabbit antibody raised against MTF (see Materials and Methods). The results (Figure 4B) show no significant differences in the levels of expression of MTF in cells carrying the wild type or the mutant MTF genes (lanes 2−6). The amount of endogenous MTF in extracts of cells expressing MTF from the chromosomal copy of the MTF gene is too little to be detected under the conditions used (lane 1).

For a more quantitative analysis of the relative activity of the various MTF genes in suppression of the formylation defect of the Mi:2/5 tRNA, the extracts were assayed for CAT activity. To correct for possible fluctuations in copy number of the pRSVpCATam1.2.5 plasmid, extracts were also assayed for β-lactamase activity, and the CAT activity in each extract was normalized to the β-lactamase activity

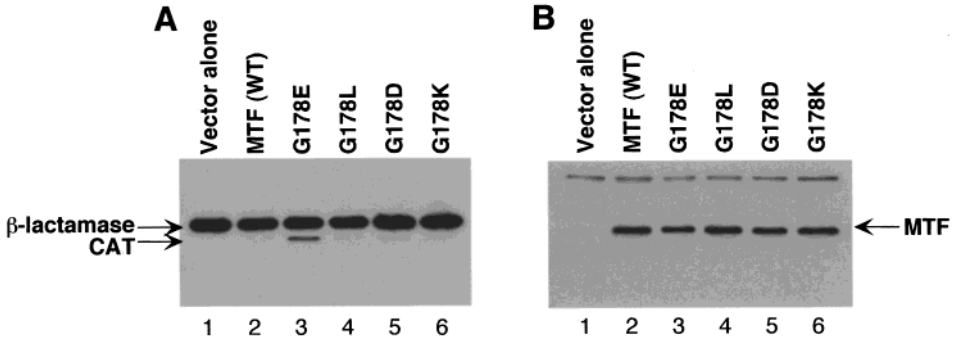

FIGURE 4: Immunoblot analysis of CAT and β-lactamase levels (A) and MTF levels (B) in extracts from *E. coli* transformants carrying the Mi:2/5 mutant tRNA genes and overproducing LysRS (lane 1) or LysRS and either wild-type MTF (lane 2) or the various G178 mutants of MTF (lanes 3−6).

Table 2: CAT, β-Lactamase, and Formylase Activities in Extracts of CA274 Transformed with pRSVp*CATam1.2.5* Carrying the Various Mutant tRNA Genes and the pACD Plasmid Carrying either the Wild-Type or the Various Mutant MTF Genes

tRNA	MTF	CAT act.[a] (units/μg)	β-lactamase act.[b] (units/μg)	normalized CAT act. (CAT/β-lactamase)	rel CAT act.[c]	formylase act. (nmol min^{-1} μg^{-1})
Mi:2/5	WT	0.92	0.39	2.3	1	3.8
	G178D	5.79	0.46	12.6	5.5	4.2
	G178E	16.46	0.36	45.7	19.9	2.3
	G178K	4.60	0.40	11.5	5.0	3.7
	G178L	2.11	0.44	4.8	2.1	4.9
Mi:2/4,5	WT	2.32	0.40	5.8	1	3.6
	G178D	11.12	0.40	27.8	4.8	3.4
	G178E	25.0	0.33	75.7	13.0	2.0
	G178K	8.18	0.40	20.4	3.5	3.5
	G178L	6.71	0.38	17.6	3.0	3.5
Mi:3/A51:U63	WT	1.10	0.40	2.7	1	4.7
	G178D	2.11	0.48	4.4	1.6	4.6
	G178E	6.62	0.45	14.7	5.4	2.8
	G178K	0.74	0.42	1.8	0.7	3.8
	G178L	1.10	0.40	2.7	1.0	4.8

[a] 1 unit of CAT activity is defined as nmoles of chloramphenicol converted to acetyl chloramphenicol in 15 min. at 37 °C. [b] The definition of a unit of β-lactamase activity is as described before (5). [c] Relative CAT activity is the ratio of normalized CAT activity in extracts from cells carrying the various mutant MTF genes divided by the normalized CAT activity in the extract carrying the wild-type MTF gene.

in the same extract (*5*). As a further control, the extracts were also assayed for MTF activity (*30*). The results (Table 2) show that cells overproducing the G178E mutant MTF contain the highest levels of CAT activity, approximately 20-fold more than cells overproducing the wild-type MTF. Cells expressing the other mutants contain lower levels of CAT activity. These results are consistent with the chloramphenicol resistance phenotype of cells carrying the various MTF genes.

The results in Table 2 (last column) also show that the higher level of CAT activity in cells expressing the G178E mutant MTF is not because these cells contain more of the mutant MTF. There is, if anything, less MTF activity in cells expressing the G178E mutant MTF. The levels of expression of the wild type and the other MTF mutants are essentially the same.

Allele Specificity of Suppression. The critical determinants for formylation in the *E. coli* initiator tRNA are the C1$\times$A72 mismatch and the G2·C71 and the C3·G70 base pairs in the acceptor stems. The G4·C69 and the G5·C68 base pairs also play some role in formylation, particularly in the context of an elongator methionine tRNA sequence background (*8*). In addition, the A11·U24 base pair in the dihydrouridine stem also plays some role in formylation (*7*, *14*). The Mi:2/5 mutant elongator methionine tRNA used in this work to select for suppressors in MTF has the critical determinants for formylation in the acceptor stem. It also has the G5·C68 base pair. However, it is lacking the G4·C69 and A11·U24 base pairs. In the context of an elongator methionine tRNA sequence background, the G4·C69 base pair is thought to be very important for formylation, k_{cat}/K_m down by a factor of 125 (*8*). To investigate whether the G178E mutant enzyme compensates for the absence of these other determinants for formylation, we have measured CAT activity in extracts of cells carrying the pRSVp*CATam1.2.5* plasmid with either the Mi:2/4/5 tRNA or the Mi:3/A51:U63 tRNA (Figure 5). The Mi:2/4/5 mutant tRNA has the G4·C69 base pair, and the Mi:3/A51:U63 mutant has the A11·U24 base pair along with the C63 to U63 mutation necessary for expression of the tRNA (*13*, *14*). These tRNAs are also aminoacylated with lysine in vivo.

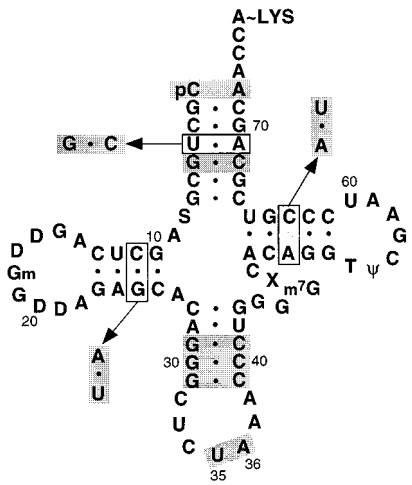

FIGURE 5: Cloverleaf structure of the mutant tRNAs, derived from Mi:2/5 tRNA, used for testing the allele specificity of suppression of the G178E mutant MTF. The Mi:2/4/5 mutant tRNA has a G4·C69 base pair in addition to the other mutations in the Mi:2/5 tRNA, and the Mi:3/A51:U63 tRNA has an A11·U24 base pair and an A51·U63 base pair in addition to the other mutations in the Mi:2/5 tRNA.

The results (Table 2) show that the fold increases in CAT activity in cells expressing the Mi:2/4/5 mutant tRNA and the various mutant MTFs, compared to wild-type MTF, are about the same as in cells expressing the Mi:2/5 tRNA. These results suggest that the G178E mutant is not compensating for the formylation defect of the Mi:2/5 tRNA because it lacks the G4·C69 base pair. Results with the Mi:3/A51:U63 mutant tRNA, similarly, suggest that the G178E mutant is not compensating for the formylation defect of the Mi:2/5 tRNA because it lacks the A11·U24 base pair. Thus, the G178E mutant compensates for the amino acid that is attached to the tRNA.

DISCUSSION

We have isolated mutations in MTF which suppress the defect in formylation of the mutant *E. coli* elongator methionine tRNA, Mi:2/5, that is aminoacylated with lysine. The suppressor mutation has a Gly178 to Glu change in

MTF. Of the two different suppressor mutants isolated, both had in common the G178E mutation. Comparison of suppressor activities of other mutants generated at the 178 position showed that the Glu178 mutant was the best suppressor.

The suppressor mutations in MTF were initially selected on the basis of the phenotypic resistance of *E. coli* carrying the mutant CAT gene and the Mi:2/5 mutant tRNA to chloramphenicol. Assays for CAT activity in extracts of *E. coli* show that the phenotypic resistance to chloramphenicol is due to an approximately 20-fold increase in CAT activity in *E. coli* overproducing the G178E mutant MTF compared to *E. coli* overproducing the wild-type MTF. This increase in CAT activity is, in turn, due to the fact that in *E. coli* overproducing the G178E mutant MTF, the Mi:2/5 mutant tRNA is formylated to the extent of ~50%, whereas in cells overproducing the wild-type MTF, there is essentially no formylation of the Mi:2/5 tRNA. These results highlight once again the importance of formylation in the activity of a tRNA in initiation of protein synthesis in *E. coli*. In vitro studies show that the G178E mutant MTF is slightly less active on the wild-type initiator Met-tRNA as substrate compared to wild-type MTF. Thus, the suppressor activity of the G178E mutant is not due to a general nonspecific increase in catalytic activity of the enzyme.

The critical determinants for formylation in the *E. coli* initiator tRNA are located in the acceptor stem and include the C1×A72 mismatch and the G2·C71 and the C3·G70 base pairs (*7*−*10*). Other determinants include the G4·C69 and the G5·C68 base pairs in the acceptor stem and the A11·U24 base pair in the dihydrouridine stem. The Mi:2/5 mutant tRNA used for selection of the suppressor mutations in MTF has the critical determinants in the acceptor stem needed for formylation; however, it lacks the G4·C69 base pair and the A11·U24 base pair. Therefore, it was possible that the G178E suppressor mutation compensates for the lack of either of these base pairs in the Mi:2/5 mutant tRNA. Our finding (Table 2) that the G178E mutant also suppresses the formylation defect of the Mi:2/4/5 and the Mi:3/A51:U63 mutant tRNAs (Figure 5) rules out these possibilities and suggests that this mutation compensates for the fact that the Mi:2/5 mutant tRNA is a very poor substrate for formylation primarily because of the amino acid lysine attached to the tRNA.

The mechanism by which the G178E mutation compensates for the strong negative effect of lysine on the Mi:2/5 mutant tRNA is not known. In the crystal structure of the complex of MTF with the *E. coli* fMet-tRNA (*20*), the side chain of methionine fits into a hydrophobic pocket surrounded by side chains of Phe14, Ala89, Pro122, Ile123, Leu136, Tyr168, and Leu171, but Gly178 is not part of the amino acid binding pocket of MTF (*34*). The crystal structure of the complex involves fMet-tRNA, the product of formylation, and not Met-tRNA, the substrate for formylation. Assuming that the position of the methionine side chain in the complex does not change between Met-tRNA and fMet-tRNA, the COO^- side chain of Glu178 would be nearby but not close enough for a direct interaction with the side chain NH_3^+ group of lysine attached to the tRNA. A possible explanation for the suppressor activity of the G178E mutant MTF is that the G178E mutation induces structural changes in the amino acid binding pocket of MTF, which somehow allow the α-amino group of the lysine attached to the Mi:2/5 tRNA to fit better into the catalytic site of MTF. Docking of lysine instead of formylmethionine in the amino acid binding pocket of MTF shows that lysine can be accommodated into the pocket except for possible steric clash between the NH_3^+ group of the lysine side chain and either the NH of the imidazole ring of His110 or the main chain NH of Gly111 (E. Schmitt and S. Blanquet, personal communication). The G178 to Glu mutation could, as indicated below, lead to rearrangements of the amino acid binding pocket of MTF such that it can more readily accommodate a lysine residue. Mutation of Gly178 to Glu results in steric clashes of the Glu side chain with side chains of either residues 136 and 154 (E. Schmitt and S. Blanquet, personal communication) or residues 138 and 152. In the crystal structure, the main chain NH of residue 137 interacts with the main chain CO of His110. Therefore, it might be speculated that the steric clash of Glu178 with residues 136 or 138 leads to movement of the main chain NH of residue 137, which in turn affects the position of the His110−Gly111 region of MTF and, thereby, leads to accommodation of lysine in the amino acid binding pocket of MTF.

ACKNOWLEDGMENT

We thank Drs. Emmanuelle Schmitt and Sylvain Blanquet for performing the docking of lysine in the crystal structure of the MTF·fMet-tRNA complex and for their kind suggestions on a possible mechanism for the suppressor activity of the G178 mutant MTF. We thank Drs. Anne Kowal and Christine Mayer of our laboratory for their help with computer simulation of the effects of the mutations in MTF. We thank Annmarie McInnis for patience, care, and enthusiasm in the preparation of the manuscript.

REFERENCES

1. Marcker, K., and Sanger, F. (1964) *J. Mol. Biol. 8*, 835−840.
2. Kozak, M. (1983) *Microbiol. Rev. 47*, 1−45.
3. Gualerzi, C. O., and Pon, C. L. (1990) *Biochemistry 29*, 5881−5889.
4. RajBhandary, U. L. (1994) *J. Bacteriol. 176*, 547−552.
5. Varshney, U., Lee, C.-P., Seong, B. L., and RajBhandary, U. L. (1991) *J. Biol. Chem. 266*, 18018−18024.
6. Dickerman, H. W., Steers, E., Jr., Redfield, B. G., and Weissbach, H. (1967) *J. Biol. Chem. 242*, 1522−1525.
7. Lee, C. P., Seong, B. L., and RajBhandary, U. L. (1991) *J. Biol. Chem. 266*, 18012−18017.
8. Guillon, J.-M., Meinnel, T., Mechulam, Y., Lazennec, C., Blanquet, S., and Fayat, G. (1992) *J. Mol. Biol. 224*, 359−367.
9. Lee, C.-P., Dyson, M. R., Mandal, N., Varshney, U., Bahramian, B., and RajBhandary, U. L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9262−9266.
10. Lee, C. P., Mandal, N., Dyson, M. R., and RajBhandary, U. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7149−7152.
11. Giege, R., Ebel, J. P., and Clark, B. F. C. (1973) *FEBS Lett. 30*, 291−295.
12. Varshney, U., Lee. C. P., and RajBhandary, U. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2305−2309.
13. Li, S., Kumar, N. V., Varshney, U., and RajBhandary, U. L. (1996) *J. Biol. Chem. 271*, 1022−1028.
14. Ramesh, V., Varshney, U., and RajBhandary, U. L. (1997) *RNA 3*, 1220−1232.
15. Varshney, U., and RajBhandary, U. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1586−1590.
16. Seong, B. L., and RajBhandary, U. L (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 334−339.
17. Mandal, N., Mangroo, D., Dalluge, J. J., McCloskey, J. A., and RajBhandary, U. L. (1996) *RNA 2*, 473−482.

18. Guillon, J.-M., Mechulam, Y., Blanquet, S., and Fayat, G. (1993) *J. Bacteriol. 175*, 4507−4514.
19. Vaidyanathan, R., Gite, S., Li, Y., and RajBhandary, U. L. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13524−13529.
20. Schmitt, E., Panvert, M., Blanquet, S., and Mechulam, Y. (1998) *EMBO J. 17*, 6819−6826.
21. Yanisch-Perron, C., Viera, J., and Messing, J. (1985) *Gene 33*, 103−119.
22. Mangroo, D., and RajBhandary, U. L. (1995) *J. Biol. Chem. 270*, 12203−12209.
23. Kumar, N. V., and Varshney, U. (1994) *Curr. Sci. 67*, 728−734.
24. Guillon, J. M., Mechulam, Y., Schmitter, J. M., Blanquet, S., and Fayat, G. (1992) *J. Bacteriol. 174*, 4294−4301.
25. Mandal, N., and RajBhandary, U. L. (1992) *J. Bacteriol. 174*, 7827−7830.
26. Remy, P., Birmele, C., and Ebel, J. P. (1972) *FEBS Lett. 27*, 134−137.
27. Kahn, D., Fromant, M., Fayat, G., Dessen, P., and Blanquet, S. (1980) *Eur. J. Biochem. 105*, 485−497.
28. Miller, J. H. (1992) *A short course in bacterial genetics*, pp 293−297, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
30. Ramesh, V., Gite, S., and RajBhandary, U. L (1998) *Biochemistry 37*, 15925−15932.
31. Varshney, U., Lee, C.-P., and RajBhandary, U. L. (1991) *J. Biol. Chem. 266*, 24712−24718.
32. Normanly, J., Kleina, L. G., Masson, J.-M., Abelson, J., and Miller, J. H. (1990) *J. Mol. Biol. 213*, 719−726.
33. Hochuli, E., Döbeli, H., and Schacher, A. (1987) *J. Chromatogr. 411*, 177−184.
34. Schmitt, E., Blanquet, S., and Mechulam, Y. (1996) *EMBO J. 15*, 4749−4758.

BI000625+