Anticodon Sequence Mutants of *Escherichia coli* Initiator tRNA: Effects of Overproduction of Aminoacyl-tRNA Synthetases, Methionyl-tRNA Formyltransferase, and Initiation Factor 2 on Activity in Initiation†

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ABSTRACT: Anticodon sequence mutants of *Escherichia coli* initiator tRNA initiate protein synthesis with codons other than AUG and amino acids other than methionine. Because the anticodon sequence is, in many cases, important for recognition of tRNAs by aminoacyl-tRNA synthetases, the mutant tRNAs are aminoacylated in vivo with different amino acids. The activity of a mutant tRNA in initiation in vivo depends on (i) the level of expression of the tRNA, (ii) the extent of aminoacylation of the tRNA, (iii) the extent of formylation of the aminoacyl-tRNA to formylaminoacyl-tRNA (fAA-tRNA), and (iv) the affinity of the fAA-tRNA for the initiation factor IF2 and the ribosome. Previously, using *E. coli* overproducing aminoacyl-tRNA synthetases, methionyl-tRNA formyltransferase, or IF2, we identified the steps limiting the activity in initiation of mutant tRNAs aminoacylated with glutamine and valine. Here, we have identified the steps limiting the activity of mutant tRNAs aminoacylated with isoleucine and phenylalanine. The combined results of experiments involving a variety of initiation codons (AUG, UAG, CAG, GUC, AUC, and UUC) provide support to the hypothesis that the ribosome·fAA-tRNA complex can act as an intermediate in initiation of protein synthesis. Comparison of binding affinities of various fAA-tRNAs (fMet-, fGln-, fVal-, fIle-, and fPhe-tRNAs) to IF2 using surface plasmon resonance supports the idea that IF2 can act as a carrier of fAA-tRNA to the ribosome. Other results suggest that the C1xA72 base pair mismatch, unique to eubacterial and organellar initiator tRNAs, may also be important for the binding of fAA-tRNA to IF2.

Of the two classes of methionine tRNAs found in all organisms, the initiator is used for the initiation of protein synthesis, whereas the elongator is used for the insertion of methionine into internal positions (*1*, *2*). In eubacteria, mitochondria, and chloroplasts, the initiator tRNA is used as formylmethionyl-tRNA (fMet-tRNA). Following aminoacylation, the initiator methionyl-tRNA (Met-tRNA) is formylated to fMet-tRNA (*3*) by methionyl-tRNA formyltransferase (MTF).[1] The fMet-tRNA then binds to the 30S ribosome in a reaction facilitated by the initiation factors IF1, IF2, and IF3 to form a 30S·mRNA·fMet-tRNA initiation complex (*4*, *5*). IF2 is thought to help the ribosome select fMet-tRNA over other tRNAs by virtue of the fact that, other than peptidyl-tRNAs on the ribosome, fMet-tRNA is the only tRNA in the cell that carries an N-acyl amino acid (*6*). How IF2 works and whether IF2 acts as a carrier of fMet-tRNA to the ribosome is not established. Also not established is whether the 30S ribosome obligatorily binds first to mRNA and then to fMet-tRNA or vice versa.

Because of their unique function, initiator tRNAs have many properties that are distinct from those of elongator tRNAs. Using in vitro and in vivo approaches, we have previously identified the sequence and/or structural elements in the *Escherichia coli* initiator tRNA important for specifying these distinctive properties (*5*). The strategy for the in vivo approach was based on the use of a mutant initiator tRNA (U35A36) carrying an anticodon sequence change from CAU to CUA. This anticodon mutation allows assessment of the initiator activity of the mutant tRNAs in vivo by measuring chloramphenicol acetyltransferase (CAT) activity in extracts of cells carrying a mutant reporter CAT gene (*CATam1.2.5*), with UAG as the initiation codon (*7*). Because of the anticodon sequence change, the mutant initiator tRNA is now aminoacylated with glutamine (*7*, *8*). Consequently, CAT protein synthesis is initiated with formylglutamine (fGln). In attempts to determine if any of the steps of aminoacylation, formylation, binding of the fMet-tRNA to IF2 or to the ribosomal P site were limiting for initiation, we studied the expression of the reporter CAT protein in cells overproducing glutaminyl-tRNA synthetase (GlnRS), MTF, or IF2. Surprisingly, in cells overproducing GlnRS and IF2, but not GlnRS alone, the amount of CAT protein made was almost 9 times more than that in cells carrying the wild-type CAT gene and the wild-type initiator tRNA (*9*). These and other results on binding of fGln-tRNA

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; MTF, methionyl-tRNA formyltransferase; fAA-tRNA, formylaminoacyl-tRNA.

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to IF2 in vitro suggested that the limiting step in initiation was the poor binding of fGln-tRNA to IF2 (*10*). Similar experiments with a CAU to GAC (G34C36) anticodon mutant of the *E. coli* initiator tRNA and a *CATGUC1.2.5* gene (previously called *CATV1.2.5*) carrying GUC as the initiation codon showed that the CAT activity went up in cells overproducing valyl-tRNA synthetase (ValRS) or MTF or both but not in cells overproducing IF2 (*11*). Thus, both aminoacylation and formylation of this mutant tRNA were limiting for initiation in *E. coli* but not binding of fVal-tRNA to IF2. In this case also, under conditions optimal for production of fVal-tRNA, the amount of CAT protein made from the mutant initiator tRNA and the mutant CAT gene was 5−6 times that made from the wild-type initiator tRNA and the wild-type CAT gene. These findings led to the hypotheses (i) that a ribosome-initiator tRNA complex can act as an intermediate in translation initiation and (ii) that IF2 can act as a carrier of the initiator tRNA to the ribosome (*10*, *11*).

The work described in this paper provides further support to these hypotheses. We have investigated the steps limiting the activity of two more mutant initiator tRNAs, G34 and G34A36, aminoacylated with isoleucine and with phenylalanine, respectively. We were particularly interested in the G34A36 mutant because a previous report had shown that this mutant was much less active in initiation (<5% of the activity compared to wild-type initiator tRNA) (*12*). We measured CAT activity in cells overproducing isoleucyl-tRNA synthetase (IleRS), phenylalanyl-tRNA synthetase (PheRS), MTF, or IF2. In parallel, we used acid urea gel electrophoresis of total tRNA from transformants followed by Northern blot analysis to identify the state of the tRNAs in vivo (*13*). The results show that aminoacylation, formylation, and to some extent affinity for IF2 are all limiting for activity of the G34 mutant tRNA aminoacylated with isoleucine, whereas aminoacylation and affinity for IF2 are limiting for activity of the G34A36 mutant tRNA aminoacylated with phenylalanine. Strikingly, CAT activity in extracts of cells transformed with a plasmid carrying the G34A36 mutant initiator tRNA increased by 10 fold in cells overproducing PheRS and to a value twice that in cells carrying the wild-type tRNA gene. The results of these studies, thus, provide additional support to the hypothesis that a ribosome−initiator tRNA complex can act as an intermediate in translation initiation.

Along with the in vivo work, we have measured the binding affinities of the various fAA-tRNAs (fMet-, fGln-, fVal-, fIle-, and fPhe-tRNA) to IF2 using surface plasmon resonance (SPR). We find a reciprocal relationship between the effect of overproduction of IF2 on activity of the mutant tRNA in initiation and the relative order of binding affinities of the corresponding fAA-tRNAs to IF2. These results provide further support to the hypothesis that IF2 can act as a carrier of fAA-tRNA to the ribosome. In further studies on binding of mutant initiator tRNAs to IF2 using SPR, we have found that the C1xA72 base pair mismatch, one of the most highly conserved features in eubacterial initiator tRNAs, may also be important for the binding of fMet-tRNA to IF2.

MATERIALS AND METHODS

General. Standard procedures were used for work involving recombinant DNA (*14*). DNA sequencing was carried out with the USB T7 DNA Sequenase 2.0 sequencing KIT according to the manufacturer's protocol. Restriction enzymes were purchased from New England Biolabs. Radiochemicals were obtained from NEN/Perkin-Elmer Life Sciences and Amersham Pharmacia Biotech.

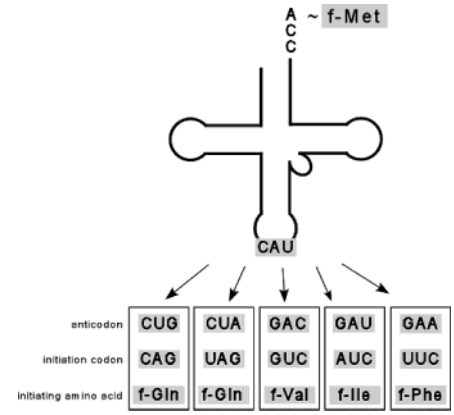

FIGURE 1: Schematic cloverleaf representation of *E. coli* fMet-tRNA^{fMet} highlighting the wild-type anticodon sequence and the fMet attached to the 3′-end. Boxed areas indicate (from top to bottom) the anticodon sequences of the mutant initiator tRNA^{fMet} used in this study, the corresponding mutant initiation codons introduced into the CAT reporter gene, and the N-terminal amino acids inserted into the CAT reporter protein.

Plasmids, Vectors, and Strains. E. coli strains CA274 (*HfrH lacZ125am trpEam*) and MC4100 [*F′araD139* Δ*(lac) U169 prsL150 relA1 deoC1 rbsR fthD5301 fruA25 λ⁻ ClpA:Kan*] were used as hosts for analysis of the activity of the various mutant initiator tRNA genes. *E. coli* strain BL21-(DE3) pLysS [*F⁻ompT hsdS$_B$ r$_B$⁻ m$_B$⁻ gal dcm (λcIts857 ind1 sam7 nin5 lacUV5-T7) pLysS (Cm^r)*] was used to express T7 promoter based expression constructs. *E. coli* DH5α *(endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 lacZYA-argF deoR*) was chosen as host for plasmid construction and propagation, *E. coli* MV1184 [*ara* Δ*(lac-proAB) rspL thi (Φ80 lacZΔM15)* Δ*(srl-recA) 306::Tn10(tet^r)/F′(traD36 proAB$^+$ lacI^q lacZΔM15)*] as host for IleRS expression, and JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ*(lac-proAB) F′ (traD36 proAB$^+$ lacI^q lacZΔM15)*] as host for IF2-α$_{His6}$ expression, respectively. The wild type or the various mutant CAT reporter genes and the tRNA^{fMet} genes are cloned into the plasmid pRSVp (*15*) (Figures 1 and 2). The plasmid pACD (*9*) was used for overproduction of GlnRS, ValRS, IleRS, MTF, and IF2. The sites used for cloning are indicated in Figure 2 (arrows). pACDMTF, pACDIF2, pACDGlnRS, pACDGlnRSIF2, pACDValRS, and pACDValRSMTF have been described before (*9*, *11*). pACDMTFIF2 was constructed by inserting the genes *fms-fmt* (excised from pGFIBI by digestion with *Pvu*II) into the blunt-ended *Eco*RI site of pACDIF2. The *ileS* gene was amplified from JM109 genomic DNA using 5′-TCCCGAATTCATTGCGCGTGATGAATTAACC-3′ as the forward primer and 5′-AATGGAATTCAAAAAACGGAGCATGCTCCGGTTGAACAGATCGATTGACTC-3′ as the reverse primer. Both primers add *Eco*RI sites for cloning, and the 3′ primer inserts the primary sequence for part of a transcription termination signal from the *valS* gene. The PCR fragment was digested with *Eco*RI and ligated to pACD linearized with the same enzyme. pACDIRSMTF was constructed by inserting the genes *fms-fmt* (excised from

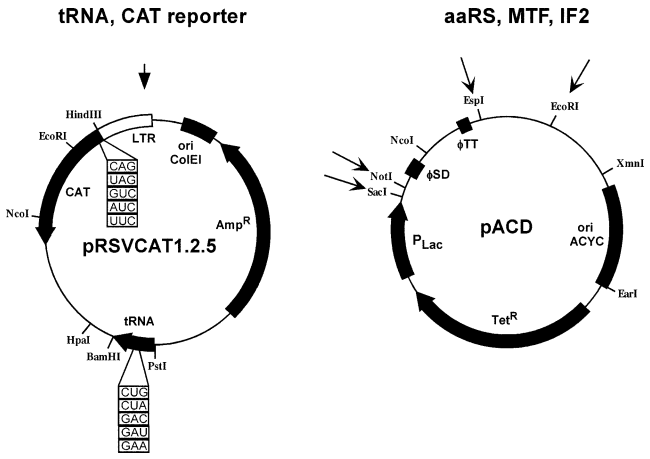

FIGURE 2: Plasmids used for in vivo studies of the activity of initiator tRNA mutants. (left) The pRSV*CAT1.2.5* plasmid used for expression of the CAT reporter gene and the initiator $tRNA^{fMet}$. The mutations of the start codon within the CAT gene and mutations of the anticodon sequence within the $tRNA^{fMet}$ gene are indicated. (right) The pACD plasmid used for the expression of the various aminoacyl-tRNA synthetases, MTF and IF2.

pGFIBI with *Pvu*II digestion) into the blunt-ended *Sac*I site of pACDIRS. *pheS* and *pheT* genes encoding, respectively, the α and β subunits of PheRS ($\alpha 2\beta 2$) were released from plasmid pB1 (*16*) by digestion with *Stu*I and *Hin*dIII and inserted into pRSV*CATUUC1.2.5/trnfMG34A36* linearized with *Hpa*I. The insert also contains the entire wild-type control region for transcription and translation of the *phe* operon, i.e., two natural promoters and one strong termination signal, the leader peptide pheM, and two small ribosomal proteins L20 and L35.

Purified Proteins and Cell-Free Extracts. His-tagged *E. coli* MTF was purified by Dr. Sadanand Gite, *E. coli* methionyl-tRNA synthetase (MetRS) was purified by Dr. Mike Dyson, and *E. coli* S100 extract enriched in ValRS was prepared by Dr. Xin-Qi Wu. An S100 extract enriched in GlnRS was prepared from *E. coli* BL21(DE3) pLysS transformed with pET3•glnS provided by Dr. Dieter Söll, Yale University. A similar extract enriched in IleRS was prepared from *E. coli* MV1184 transformed with plasmid pKS21 provided by Dr. Paul Schimmel, Scripps Institute (*17*). An S30 extract enriched with PheRS was prepared from *E. coli* CA274 transformed with plasmid pB1 provided by Dr. Mathias Springer, Institut de Biologie Physico-Chimique (*16*). All S100 and S30 extracts were freed of tRNAs by DEAE-cellulose chromatography.

Growth of Cells and Preparation of Cell Extracts. Transformants of *E. coli* strain CA274 or MC4100/ClpA$^-$ were picked and used to inoculate 5 mL of LB medium supplemented with antibiotics as necessary. The concentration of ampicillin, tetracyclin and kanamycin was 100−200, 10, and 50 μg/mL, respectively. Cells were grown at 37 °C until late-log phase (A_{600} of 1.6−1.8) was reached and 1 mL of each of the cultures was used to prepare total cell extract as described (*18*). The protein concentration of the extract was determined by the Bio-Rad dye-binding assay using BSA as standard. Extracts were then diluted to a concentration of 1 mg/mL using TME buffer (25 mM Tris-HCl pH 8.0, 2 mM β-mercaptoethanol, 1 mM EDTA), adjusted to 0.5 mg/mL with 2 × protein storage buffer (70% glycerol, 20 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 200 mM NaCl) and stored at −20 °C.

Assay for CAT Activity. CAT activity in cell extracts was assayed as described (*18*). To minimize the effect of fluctuations in the copy number of the plasmid bearing the CAT gene, the specific activities of CAT were normalized to the specific activities of β-lactamase in the same extract.

Assay for β-Lactamase. A 500 μg/mL stock solution of Nitrocefin (3-(2,4-dinitrostyryl)-(6*R*,7*R*)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer; Calbiochem) was prepared in 0.1 M sodium phosphate buffer pH 7.0 and stored according to the supplier's recommendation. Shortly before the assay, the Nitrocefin stock was diluted 10-fold in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (working concentration 50 μg/mL Nitrocefin). A 1 μg sample of protein extract was mixed with 1 mL of Nitrocefin solution and incubated at room temperature for 12−15 min. The reaction was stopped by addition of 110 μL of a 10% SDS solution and absorbance was read at 486 nm against a reagent blank.

Analysis of Aminoacylation and Formylation Levels of tRNA in Vivo. Total tRNA from *E. coli* was isolated under acidic conditions by the guanidine thiocyanate-phenol-chloroform method (*19*) (TriReagent; Molecular Research Center, Cincinnati). Typically, the pellet of 3 mL of bacterial culture was extracted with 500 μL of the reagent. After precipitation with 2-propanol, the RNA pellet was washed with 75% ethanol and dissolved in 20−30 μL of 10 mM sodium acetate pH 5.2, 1 mM EDTA. An aliquot containing 0.02−0.05 OD_{260} of RNA was subjected to electrophoresis on a 6.5% polyacrylamide, 7 M urea gel at pH 5.0 and 4 °C. The RNA was transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech), and mutant tRNAs were detected by hybridization with ^{32}P-labeled oligonucleotides complementary to the anticodon stem and loop sequences (*13*).

Western Blot Analysis. After separation of the proteins by electrophoresis on a 15% SDS-polyacrylamide gel, transfer of proteins to an Immobilon-P membrane (Millipore) was carried out in a Novex Mini Cell II Blot module, following protocol II of the supplier's manual. Antibodies utilized were rabbit anti-CAT and rabbit anti-β-lactamase (both 5′−3′ Inc.) (*18*). The secondary antibody was anti-rabbit IgG (Amersham Pharmacia Biotech), and detection of the antibody-coupled horseradish peroxidase was performed with the ECL oxidase/luminol reagents (NEN/Perkin-Elmer Life Sciences).

Purification of Initiator tRNAs. Wild-type and mutant initiator tRNAs were expressed in *E. coli* B105 (*20*) transformed with pUC13*trnfM* or pRSV*CAT* plasmids carrying the mutant initiator tRNA genes, respectively. The initiator tRNA species used throughout is the $tRNA_2^{fMet}$ species, which differs from $tRNA_1^{fMet}$ by a single base change at position 46. Total RNA was isolated by phenol extraction and initiator tRNA was purified as follows: wild-type initiator tRNA was purified by DEAE-Sephadex A50 chromatography using a linear gradient of 0.375 M NaCl and 0.008 M $MgCl_2$ to 0.5 M NaCl and 0.016 M $MgCl_2$ in 20 mM Tris-HCl pH 7.5 (*21*). Mutant initiator tRNAs were purified by electrophoresis of total tRNA on 15% native polyacrylamide gels (*22*). The mutant initiator tRNAs were essentially homogeneous except for the G34A36 mutant, which was >90% homogeneous.

Preparative Aminoacylation and Formylation of Initiator tRNAs. (A) Wild-Type Initiator tRNA^{fMet}. This was carried out in two steps. First, 7 A_{260} units of purified tRNA^{fMet} were incubated in 2.4 mL of a mixture containing aminoacylation buffer A (150 mM NH_4Cl, 10 mM $MgCl_2$, 10 μg/mL BSA, 0.1 mM EDTA, 20 mM imidazole pH 7.6), 1.7 mM ATP, 83 μM L-methionine, and 30 μg of MetRS$_{His6}$. Incubation was at 37 °C for 10 min. Then, 45 μg of MTF were added together with N^{10}-formyltetrahydrofolate (f-THF) solution (330 μM final concentration). Incubation was continued at 37 °C for 8 min more. The incubation mixture was then extracted three times with phenol/chloroform (equilibrated with 10 mM sodium acetate pH 5.2), followed by two extractions with chloroform. The tRNA from the final aqueous supernatant was precipitated by addition of sodium acetate (pH 5.2) to 0.3 M followed by 2 volumes of ethanol. After precipitation, the tRNA was dissolved in 10 mM sodium acetate (pH 5.2) and dialyzed for 3 × 1 h against 10 mM sodium acetate (pH 5.2) and 1 M NaCl, and then for 1 h against 10 mM sodium acetate (pH 5.2), and finally for 2 h against 5 mM sodium acetate (pH 5.2). After dialysis the tRNA was precipitated again with ethanol. Shortly before use, the tRNA was collected by centrifugation and dissolved in 5 mM sodium acetate buffer (pH 5.2), so that the final tRNA concentration was 100 μM.

(B) U35A36 Mutant Initiator tRNA^{fMet}. 4 A_{260} units of purified mutant tRNA^{fMet} were incubated in 1 mL of a mixture containing aminoacylation buffer B (30 mM HEPES−KOH pH 7.5, 10 mM $MgOAc_2$), 2 mM ATP, 480 μM L-glutamine, 5 mM DTT, and 200 μg of S100 extract enriched in GlnRS. Incubation was at 37 °C for 40 min. The formylation reaction was carried out with 18 μg of MTF and 330 μM f-THF for 10 min.

(C) G34C36 Mutant Initiator tRNA^{fMet}. 2 A_{260} units of purified mutant tRNA^{fMet} were incubated in 0.5 mL of a mixture containing aminoacylation buffer A, 2 mM ATP, 200 μM L-valine, 4 mM DTT, and 60 μg of S100 extract enriched in ValRS. Incubation was at 37 °C for 30 min. Formylation reaction was carried out with 10 μg of MTF and 330 μM f-THF for 10 min.

(D) G34 Mutant Initiator tRNA^{fMet}. One A_{260} sample of purified mutant tRNA^{fMet} was incubated in 0.5 mL of a mixture containing aminoacylation buffer B, 2 mM ATP, 100 μM L-isoleucine, 5 mM DTT, and 96 μg of S100 extract enriched in IleRS. Incubation was at 37 °C for 30 min. Formylation was carried out with 25 μg of MTF and 330 μM f-THF for 10 min.

(E) G34A36 Mutant Initiator tRNA^{fMet}. A 0.5 A_{260} sample of purified mutant tRNA^{fMet} was incubated in 0.5 mL of a mixture containing aminoacylation buffer B, 2 mM ATP, 150 μM L-phenylalanine, 5 mM DTT, and 175 μg S30 extract enriched in PheRS. Incubation was at 37 °C for 30 min. Formylation was carried out with 15 μg of MTF and 330 μM f-THF for 10 min.

(F) G72 Mutant Initiator tRNA^{fMet}. One A_{260} sample of purified mutant tRNA^{fMet} was incubated in 0.4 mL of a mixture containing aminoacylation buffer A, 2 mM ATP, 75 μM L-methionine, and 10 μg of MetRS. Incubation was at 37 °C for 10 min. Formylation was carried out with 26 μg of MTF and 330 μM f-THF. Because this mutant tRNA is a very poor substrate for MTF, the incubation time for the formylation reaction was extended to 30 min.

Extraction, precipitation, and dialysis of mutant fAA-tRNAs were performed as described for wild-type tRNA. The extent of aminoacylation and formylation of all species was confirmed by analytical acid urea gel electrophoresis of a small aliquot of the fAA-tRNAs followed by Northern blot analysis. The tRNAs were found to be essentially all aminoacylated and formylated (data not shown).

Cloning and Purification of E. coli Initiation Factor 2. The *infB* gene was amplified from plasmid pAA (*23*) by PCR and cloned into pQE30 (Qiagen). The resulting plasmid pQE-IF2 was used to express IF2-α of *E. coli* in frame with an N-terminal tag of $MRGSH_6$ (IF2-α_{His6}).

IF2-α_{His6} was purified as follows: A single colony of *E. coli* JM109 harboring plasmid pQE-IF2 was used to inoculate 15 mL of LB medium containing ampicillin (200 μg/mL). This preculture was grown for 9 h to late log phase and then stored at 4 °C overnight. The next day, 10 mL of the culture was used to inoculate 1 L of LB medium containing ampicillin (200 μg/mL). The culture was grown to an A_{600} of 0.55−0.6, and the expression of IF2-α_{His6} was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. At the same time, the culture was resupplemented with 200 μg/mL ampicillin. Cells were grown for 4 h more and then pelleted by centrifugation. The cell pellet (1.8 g wet weight) was resuspended in 25 mL buffer C (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.5 mM PMSF, 7 mM β-mercaptoethanol), and a protease inhibitor cocktail (Complete Mini, without EDTA; Roche Molecular Biochemicals) was added. Cells were disrupted by two passages through a chilled French pressure cell (10,000 PSI), and cell debris was removed by centrifugation at 10 000*g* for 30 min.

Purification of IF2-α_{His6} was carried out using the Talon Metal Affinity resin (Clontech) according to the manufacturer's recommendations for "Batch-gravity flow purification". The clarified lysate was added to 20 mL of a Talon-slurry (50% in buffer C) and nutated for 30 min at room temperature. The resin was washed three times with 5 volumes of buffer C; then the slurry was transferred to a 10 mL disposable column. The resin was again washed with 50 bed volumes of buffer C + 10 mM imidazole (pH 7.5), and bound protein was eluted with buffer C containing 50 mM imidazole. The column was operated under gravity flow at 20 °C, fractions (5 mL) were collected, the A_{280} was read, and the peak fractions were rebound to Talon resin and eluted as described. Fractions were analyzed by SDS−PAGE. At this stage IF2-α_{His6} was >90% pure, with two contaminating bands of about 30−35 kDa. Both of them were proteolytic fragments of IF2-α_{His6} and contained the $MRGSH_6$ tag, as detected by immunoblot analysis. Most of these contaminants were removed by passing the pooled fractions repeatedly through a Centricon MWCO50 (50 kDa cut off) filtration unit. IF2-α_{His6} was concentrated by dialysis against 20 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5 mM PMSF, 8 mM β-mercaptoethanol, 50% glycerol and stored at −20 °C.

Real-Time Binding Assays Using Surface Plasmon Resonance (SPR). SPR experiments were performed on a Biacore 3000 system (Biacore AB). All assays were run at 25 °C. IF2-α_{His6} was immobilized directly onto a CM5 sensor chip surface activated by N-hydroxysuccinimide and *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide using standard coupling chemistry according to the manuals provided by Biacore.

Table 1: Effect of Overproduction of aaRS, MTF, IF2, aaRS + MTF, MTF + IF2, and aaRS + IF2 on the Relative CAT Activities in CA274 Cells Carrying the U35A36, G34C36, G34, G34A36, or U35G36 Mutant tRNA Gene along with the Mutated CAT (UAG, GUC, AUC, UUC, or CAG start) Gene

start codon (initiation with fAA)	initiator tRNA anticodon	vector alone	aaRS	MTF	IF2	aaRS + MTF	MTF +IF2	aaRS + IF2
AUG (fMet)	wild type	100 (set)	84	104	64	105 ± 9	66 ± 5	−
UAG (fGln)	CUA	78 ± 5	121 ± 4	104	274 ± 30	125	209 ± 6	452 ± 9
GUC (fVal)	GAC	157	218	300 ± 44	135	541	343 ± 51	−
AUC (fIle)	GAU	119 ± 3	268 ± 29	322 ± 18	148 ± 10	503 ± 25	622 ± 33	−
UUC (fPhe)	GAA	20 ± 2	198 ± 24	20 ± 1.3	41 ± 5.3	179 ± 31	47 ± 14	272 ± 19
CAG (fGln)	CUG	43 ± 2	40 ± 9	−	201 ± 60	−	251 ± 29	−

[a] The CAT activity obtained with the wild-type CAT gene + empty pACD vector is set as 100%. The CAT levels have been normalized to β-lactamase levels in the extracts. Numbers are given as average values of at least two independent sets of experiments, ± average deviation. CA274 with PheRS+IF2 grows extremely slowly at 37 °C. Specific CAT activity in wild-type CAT extracts was 1−1.3 units (conversion of 1 nmol chloramphenicol × min^{-1} × μg^{-1}).

IF2-α_{His6} was injected at a concentration of 50 nM in coupling buffer (5 mM maleate pH 6.0) to a final response level of 1600−2500 resonance units (RU). All nonreacted carboxyl groups were blocked by treatment with ethanolamine. A flow cell, which was activated and deactivated in parallel, was used as blank reference. For each tRNA species, a new IF2 surface was prepared. The activity of each surface was confirmed by one injection of wild-type tRNA.

Real-time binding assays were performed using binding buffer [20 mM Tris-HCl pH 7.4, 20 mM NaCl, 1 mM $MgCl_2$, 0.001% (v/v) P20 surfactant] at a constant flow-rate of 5 μL/min. tRNAs were diluted from 100 μM stocks into binding buffer and were injected at various concentrations across the IF2-surface. Association phase was monitored for 4 min, and dissociation was initiated by replacing the tRNA with binding buffer and was monitored for at least 30 min. Sensorgrams were corrected for nonspecific binding of the tRNA to the sensor surface and bulk refractive index changes by subtracting the blank reference cell. Data evaluation was performed using the Evaluation Software Version 3.0.2 from Biacore.

RESULTS

Mutant Initiator tRNAs and CAT Reporter Genes Used in This Study. By using anticodon sequence mutants of *E. coli* initiator tRNA, which are recognized by aminoacyl-tRNA-synthetases (aaRS) other than MetRS, along with the corresponding mutant CAT reporter genes, we have established systems to initiate protein synthesis with amino acids other than formylmethionine (fMet), for example, formylglutamine (fGln), formylvaline (fVal), formylisoleucine (fIle), and formylphenylalanine (fPhe). Figure 1 shows a schematic representation of the initiator tRNA from *E. coli*. The main body of the $tRNA^{fMet}$ was kept constant; only bases in the anticodon sequence were mutated. Correspondingly, the start codon of a CAT reporter gene was changed as indicated. The amino acid, which is likely to be inserted into the N-terminal position of the CAT protein (*12*), is given in the last row of each column. In addition to the mutated start codon, each CAT gene has changes in the second and fifth codons, which were previously introduced to remove weak secondary sites of initiation (*7*). Synthesis of the CAT protein is, therefore, strictly dependent on the presence of a mutant initiator tRNA with the complementary anticodon sequence. These additional mutations have also been introduced into the wild-type CAT gene. The reporter genes are thus named *CATAUC1.2.5*, *CATGUC1.2.5*, *CATUUC1.2.5*, *CATCAG1.2.5*, etc.; to indicate the mutated codons, for example, *CATAUC1.2.5* indicates the CAT gene with the AUC start codon. *CAT2.5* is the designation of the CAT gene with the wild-type AUG initiation codon.

Figure 2 shows the two-plasmid system used to assay for activity of the mutant initiator tRNAs in initiation in vivo and to study the effect of overexpression of various proteins. The CAT reporter gene (wild-type or mutant) and the initiator tRNA gene (wild-type or mutant) were carried on a pRSVp vector. The aaRS, MTF, and IF2 genes were cloned into a pACD vector.

Work on mutant initiator tRNAs initiating protein synthesis with fGln from a UAG initiation codon (*9*, *24*) and fVal from a GUC initiation codon (*11*) has been described before. Depending upon the mutant tRNA, overproduction of the aaRS, MTF or IF2 was shown to result in large increases in amounts of CAT reporter protein produced in vivo. In fact, the levels of CAT protein expressed using either UAG or GUC as initiation codons substantially exceeded those using the normal AUG initiation codon. Preliminary data had also indicated that this might be true for initiation with fIle from the AUC start codon (*11*). To confirm and extend these studies, we have now performed a more detailed analysis of initiation with fIle (G34 mutant initiator tRNA) and included another mutant initiator tRNA (G34A36), which is aminoacylated with phenylalanine. We have also repeated most of the previous experiments with the U35A36 and G34C36 mutant initiator tRNAs, so that the results presented in this study are obtained under the same experimental conditions. Furthermore, to examine the importance of the nature of the codon/anticodon pairs on levels of CAT protein produced, we have constructed a CAT gene mutant with CAG as the initiation codon instead of the UAG used before. The corresponding U35G36 mutant tRNA is also aminoacylated with glutamine like the U35A36 mutant (*8*).

Initiation of Protein Synthesis from an AUC Initiation Codon Using the G34 Mutant Initiator tRNA Aminoacylated with Isoleucine: Effect of Overexpression of IleRS, MTF, and IF2 on CAT Protein Levels. Table 1 shows the relative CAT activities in cells carrying the mutant initiator tRNA genes and the mutant CAT genes. The CAT activity obtained from coexpression of wild-type pRSV*CAT2.5* plus empty pACD vector was set at 100%. The activity in cells carrying

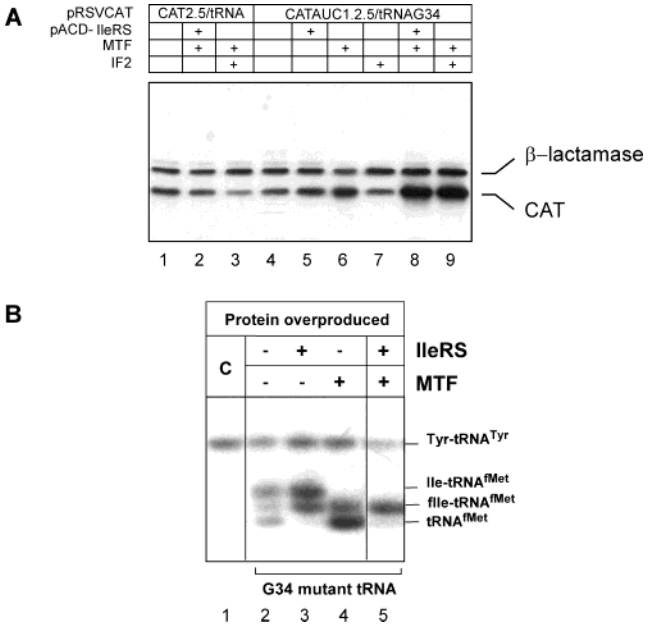

FIGURE 3: (A) Immunoblot analysis of CAT and β-lactamase levels in cell extracts from *E. coli* CA274 transformants carrying either the pRSV*CAT2.5/trnfM* (wild-type initiator tRNA) or pRSV*CAT-AUC1.2.5/trnfMG34* plasmid in combination with the pACD vector containing the genes encoding IleRS, MTF, IF2, IleRS plus MTF, or MTF plus IF2. (B) Acid urea gel analysis of the G34 mutant initiator tRNA isolated under acidic conditions from CA274 transformants overproducing IleRS, MTF, or both. Following transfer of the tRNAs from the gel to a membrane, the tRNAs were detected by hybridization to a probe specific for the G34 mutant initiator tRNA and to a probe specific for *E. coli* $tRNA^{Tyr}$.

the mutant *CATAUC1.2.5* gene, together with the G34 mutant initiator tRNA is comparable (119%) to cells carrying the corresponding wild-type genes. Overproduction of IleRS, MTF, or IF2 all led to increases in CAT activity. In cells overproducing IleRS or MTF alone, CAT activity goes up by 2.2- and 2.7-fold, respectively, whereas in cells overproducing both, CAT activity goes up by 4.2-fold. Similarly, in cells overproducing IF2 alone, CAT activity goes up slightly (1.2-fold), whereas, in cells overproducing MTF and IF2, CAT activity goes up 5.2-fold. These results suggest that aminoacylation, formylation and to some extent affinity for IF2 are all limiting for activity of the G34 mutant initiator tRNA in initiation.

Immunoblot analyses on cell extracts confirm the above results based on enzymatic assay and show that the increased CAT activities are due to increased amounts of CAT protein in the cells (Figure 3A). While the amounts of β-lactamase, an internal standard, remain quite constant, there is clearly much more CAT protein in those extracts which had shown highest CAT activity, for example, in cells overproducing IleRS, MTF, IleRS, and MTF or MTF and IF2 (Figure 3A, lanes 5, 6, 8, and 9).

To determine the effect of overproduction of IleRS and MTF on the state of the G34 mutant initiator tRNA in *E. coli*, total RNA isolated from CA274 transformants expressing the mutant tRNA was separated on an acid urea polyacrylamide gel (*13*). Uncharged, aminoacylated, and formylated forms of the G34 mutant initiator tRNA were detected by Northern blot hybridization using a probe complementary to the G34 mutant initiator tRNA and quantitated by PhosphorImager analysis. The results show that the probe used is specific and does not hybridize to wild type initiator tRNA (Figure 3B, lane 1), even though the two tRNAs differ by only one nucleotide. In cells not overproducing any of the enzymes, ~42.5% of the mutant initiator tRNA is aminoacylated, and ~22% is formylated (lane 2). Overproduction of IleRS results in conversion of most of the tRNA to a mixture of Ile-tRNA (49.1%) and fIle-tRNA (42.1%) (lane 3), whereas overproduction of MTF leads to formylation of all of the Ile-tRNA available to fIle-tRNA (lane 4). Overproduction of both IleRS and MTF leads to the conversion of essentially all of the G34 mutant tRNA to fIle-tRNA (lane 5). Thus, as seen for the G34C36 mutant (*11*), there is a clear correlation between the extent of formylation of the mutant initiator tRNA and CAT activity in cells. The blot was also probed for endogenous $tRNA^{Tyr}$ as an internal control. The results show that this elongator tRNA is essentially quantitatively aminoacylated in all samples.

Initiation of Protein Synthesis from a UUC Initiation Codon Using the G34A36 Mutant Initiator tRNA Aminoacylated with Phenylalanine: Effect of Overexpression of PheRS, MTF, and IF2 on CAT Protein Levels. Similar experiments were performed with the G34A36 mutant initiator tRNA and the *CATUUC1.2.5* gene. Results in Table 1 show that unlike most other anticodon mutant initiator tRNAs studied, CAT activity in cell extracts with the G34A36 mutant is much less (20%) than CAT activity in extracts of transformants carrying the wild-type CAT gene. However, overproduction of PheRS leads to a major increase in CAT activity by a factor of 10 corresponding to ~200% of the set value for wild-type tRNA. Overproduction of MTF has no effect, whereas overproduction of IF2 results in a doubling of CAT activity to ~40% of wild type. The overproduction of PheRS and MTF yields the same CAT activity as PheRS alone, whereas overproduction of PheRS and IF2 yields slightly more CAT (272% of that obtained with the wild-type CAT gene). These results suggest that aminoacylation of the G34A36 mutant initiator tRNA is a major limiting factor in its activity in initiation of protein synthesis with affinity for IF2 also playing a significant but a less important role.

The results of immunoblot analysis confirm the results of assay for CAT activity. There is very little CAT protein in extracts made from cells not overexpressing any of the proteins (Figure 4A, lane 2), whereas CAT protein levels go up dramatically in cells overexpressing PheRS (lanes 3, 6, and 7) and to some extent in cells overproducing IF2 (lane 5).

In addition to *E. coli* CA274, the experiments described above were also carried out in *E. coli* MC4100, a ClpA protease negative strain. This is to rule out the possibility that the rather low levels of CAT activity in extracts of cells not overproducing PheRS or other proteins are due to instability in vivo of proteins initiated with fPhe. According to the "N-end rule" (*25*), which relates the half-life of a protein to the identity of its amino-terminal residue, proteins carrying phenylalanine at the N-terminus are extremely unstable with a half-life of less than 2 min. The presence of phenylalanine at the N-terminus is thought to trigger a ClpA protease-dependent degradation pathway in *E. coli*. Figure 4A, bottom panels, shows the results of immunoblot analysis of the MC4100 strain. These are essentially identical to those

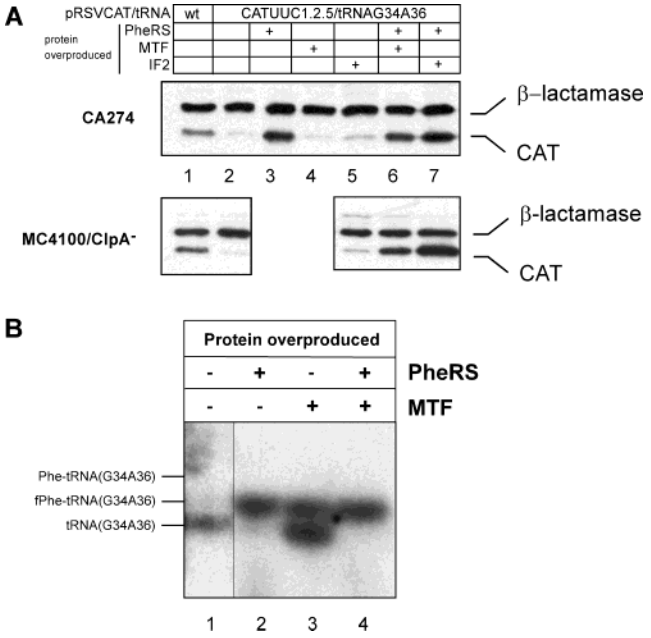

FIGURE 4: (A) Immunoblot analysis of CAT and β-lactamase levels in extracts from *E. coli* transformants. Extracts were prepared from *E. coli* CA274 (top) and MC4100/ClpA$^-$ (bottom) harboring either the pRSV*CAT2.5*/*trnfM* (tRNA wt) or the pRSV*CATUUC1.2.5*/*trnfMG34A36* plasmid in combination with the pACD vector containing the genes encoding PheRS, MTF, IF2, IleRS plus MTF, or MTF plus IF2. (B) Acid urea gel analysis of the G34A36 mutant initiator tRNA isolated under acidic conditions from CA274 transformants overproducing PheRS, MTF, or both. Northern blot analysis was carried out as described above in the legend to Figure 3, except that the probe used was specific for the G34A36 mutant initiator tRNA; weak bands corresponding to Phe-tRNA and fPhe-tRNA are not seen in lane 1 because less material was applied. The expected mobility of Phe-tRNA and fPhe-tRNA is based on the use of markers generated by in vitro aminoacylation and formylation (data not shown).

obtained from CA274 extracts (top panel). Thus, there is no significant ClpA-dependent degradation of the CAT reporter protein initiated with fPhe. It is not known whether the peptide deformylase enzyme, which removes formyl group from fMet at the N-terminus of nascent proteins, removes the formyl group from fPhe. Therefore, one possible reason for the stability of the CAT protein initiated with fPhe is that phenylalanine at the N-terminus is blocked by the formyl group.

Acid urea gel electrophoresis of tRNA followed by Northern blot analysis (Figure 4B) shows that CAT activity in extracts correlates with the extent of aminoacylation and formylation of the G34A36 mutant initiator tRNA in vivo. Without overproduction of PheRS, the tRNA is mostly uncharged. When PheRS (alone or in combination with MTF) is overproduced in the cells, the G34A36 mutant initiator tRNA is almost quantitatively converted to fPhe-tRNA. Thus, the mutant tRNA is a very poor substrate for PheRS but a good substrate for MTF. These data are consistent with results of in vitro experiments, which indicated that this mutant initiator tRNA which lacks some of the important determinants for *E. coli* PheRS is likely to be a poor substrate for *E. coli* PheRS (*26*, *27*), but a reasonably good substrate for the formylating enzyme (*28*).

Initiation of Protein Synthesis from a CAG Initiation Codon Using the U35G36 Mutant Initiator tRNA Aminoacylated with Glutamine: Effect of Overexpression of GlnRS, MTF, and IF2 on CAT Protein Levels. The mutant initiator tRNAs studied so far are aminoacylated with glutamine, valine, isoleucine, and phenylalanine. We wished to compare the activity in initiation of mutant tRNAs which are aminoacylated with the same amino acid but which have different anticodon sequences and, therefore, initiate protein synthesis with different initiation codons. For this, we have used the U35A36 and U35G36 mutant initiator tRNAs, which are both aminoacylated with glutamine but initiate protein synthesis with UAG and CAG, respectively. In view of the striking effect of overproduction of IF2 on activity of the U35A36 mutant initiator tRNA, we were particularly interested in knowing whether the U35G36 mutant initiator tRNA would also behave similarly.

Table 1 shows the results. CAT activity in extracts of cells containing the U35G36 mutant initiator tRNA is slightly lower than in extracts of cells carrying the U35A36 mutant tRNA. Interestingly, as for the U35A36 mutant, overproduction of IF2 leads to a significant increase in CAT levels. There is only a small effect of overproduction of MTF and IF2 on CAT activity compared to overproduction of IF2 alone, suggesting that, like the U35A36 mutant initiator tRNA, the U35G36 mutant tRNA is a good substrate for MTF.

Real-Time Measurements of Kinetic Rate Constants of IF2·fAA-tRNA Interaction Using Surface Plasmon Resonance: Importance of the Amino Acid and the Unique C1xA72 Mismatch in the Initiator tRNA. Immobilization of IF2 on the Sensor Chip. Previously, we have measured equilibrium dissociation constants of IF2·fAA-tRNA complexes involving wild-type initiator tRNA and two initiator tRNA mutants, fVal-tRNA and fGln-tRNA, and IF2 from *Bacillus stearothermophilus* using gel retardation analysis (*10*): these were found to be 1.6 μM for the wild-type, 3.5 μM for the fVal-, and 10.4 μM for the fGln-tRNA species. This observation supported the hypothesis that the amino acid plays a role in binding of formylated initiator tRNAs to IF2. In this study, to gain more insight into the interaction of *E. coli* IF2 and fAA-tRNAs, we have used surface plasmon resonance (SPR) to determine the kinetic parameters of the interaction in real-time.

We performed a series of pilot experiments on various ways to immobilize IF2 (ligand) on the dextran surface of the sensor chip. Among others we tried to bind IF2 via its N-terminal poly-histidine tag onto a Ni^{2+}/NTA chip. This would give the potential of generating a directed and uniform protein surface, which can be easily regenerated. However, we found that stable binding of IF2 to the Ni^{2+}/NTA chip required a high salt environment (150 mM sodium chloride). Unfortunately, such conditions are known to weaken the interaction with tRNA (*6*). We, therefore, used direct immobilization of the protein on a CM5 sensor chip using classical amine coupling chemistry. The tRNAs (analyte) were then injected across the sensor surface at concentrations ranging from 0.1 to 10 μM. Routinely, we have immobilized between 1600 and 2500 RU of IF2 (MW ~97 kDa), and upon injection of the wild-type fMet-tRNA (MW ~30 kDa), we observed an increase in RU of about 50−80 units. If all IF2 molecules bind to tRNA, the expected R_{max} value would be ~500 RU. This means that between 10% and 16% of the immobilized IF2 molecules were active in binding. A similar observation was made by Arluison et al. (1999) (*29*) in their

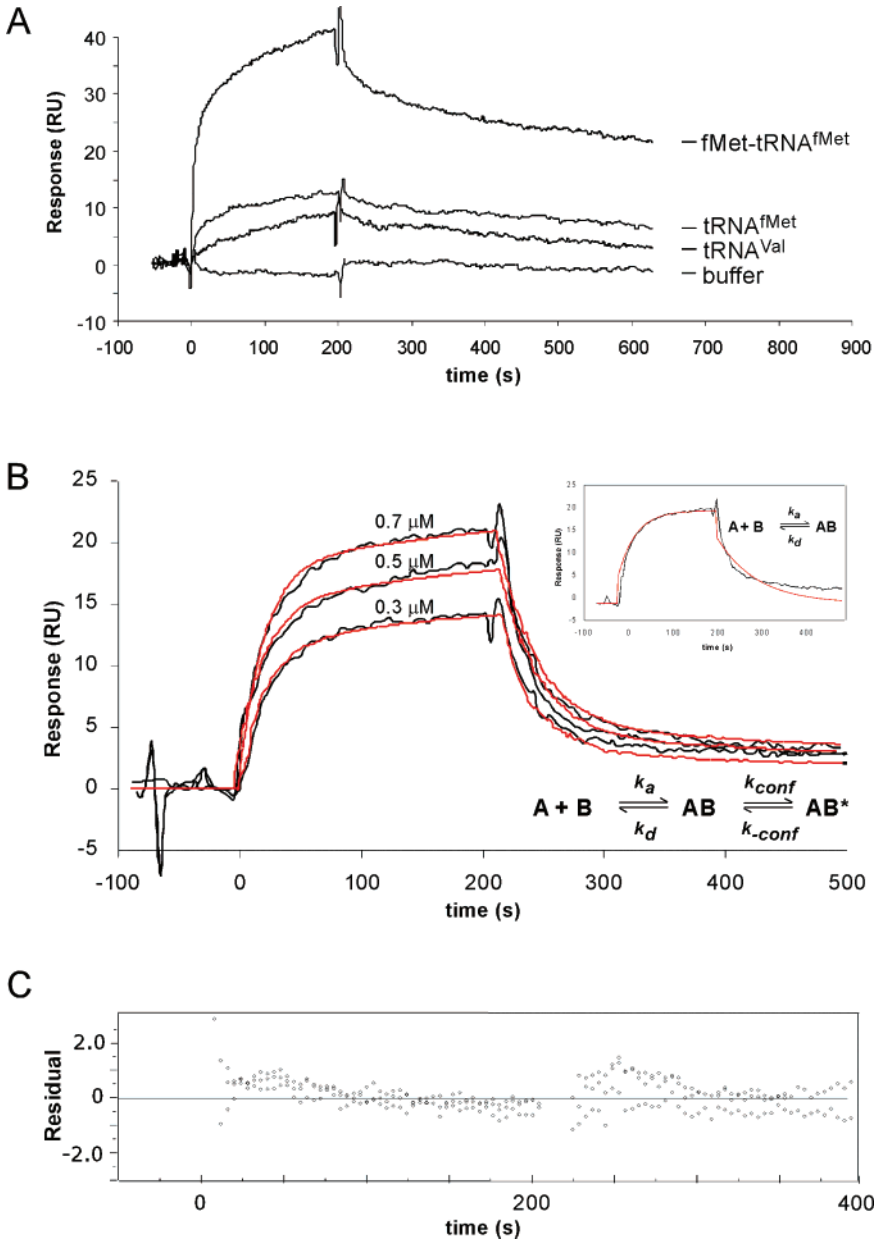

FIGURE 5: Real-time binding of *E. coli* IF2 to initiator fMet-tRNA studied using SPR. (A) Overlay plot of sensorgrams of 1 μM fMet-tRNA^{fMet}, tRNA^{fMet}, tRNA^{Val}, or buffer injected across an IF2-coupled sensor chip surface. (B) Global curve fitting for the interaction of IF2 with fMet-tRNA^{fMet} at 0.3, 0.5, and 0.7 μM. Curve fitting was executed using a model involving a conformational change of the complex. The inset shows curve fitting for the 0.7 μM injection applying a simple 1:1 Langmuir interaction model. The experimental curves are shown in black, and fitted curves are shown in red. The experiments were done in triplicate, including the use of different IF2 surfaces and yielded identical sensorgrams. (C) Distributions of residuals obtained from the global fit of B using the model involving a conformational change of the complex.

work with pseudouridine synthetase *Pus1*. One reason may be that the tRNA binding surface of a certain fraction of these immobilized tRNA binding proteins is not accessible to tRNA. There is also the possibility that the immobilization of a protein via the ϵ-amino group of some lysine residues damages the protein, e.g., by altering the tertiary structure.

tRNA Specificity of IF2 Binding. The first set of injections was performed in order to ascertain the specificity of IF2 binding to tRNA under the chosen experimental conditions. Figure 5A shows the superimposed sensorgrams of injections of wild-type initiator fMet-tRNA, uncharged wild-type initiator tRNA, uncharged elongator tRNA^{Val} (each 1 μM), and a buffer control. A signal change with a distinct association and a dissociation phase can be observed during the injection of the tRNAs, the signal of fMet-tRNA being significantly stronger than that of the uncharged tRNAs. Interestingly, uncharged initiator tRNA binds to IF2 better than uncharged elongator tRNA^{Val}, as indicated by a faster rate of association (Figure 5A), suggesting that, besides the fMet moiety, IF2 recognizes at least to some extent also the body of the tRNA. The buffer injection provides the baseline and has only the injection spikes, which are an inherent property of the injection mode.

Next, we wanted to determine the kinetic rate constants of IF2 binding toward its wild-type substrate, fMet-tRNA. Various concentrations of tRNA ranging from 0.1 to 1.5 μM were applied to the sensor chip. Figure 5B shows the global fit of three binding curves (at 0.3−0.7 μM concentrations of fMet-tRNA), which were selected for curve fitting using the Biacore evaluation software. Initially we tried to apply

Table 2: Rate Constants and Apparent Equilibrium Dissociation Constants for fMet-tRNA^{fMet} (wt) and fMet-tRNA^{fMet} (G72) Binding to *E. Coli* IF2 as Determined by SPR[a]

	k_a ($M^{-1}\cdot s^{-1}$)	k_d (s^{-1})	k_{conf} (s^{-1})	k_{-conf} (s^{-1})	K_D (M)
fMet-tRNA (wt)	2.94×10^4	0.0295	1.31×10^{-3}	1.21×10^{-3}	9.26×10^{-7}
fMet-tRNA(G72)	4.16×10^3	–	–	–	–

[a] Values were calculated from global fits of sensorgrams in Figures 5 and 7.

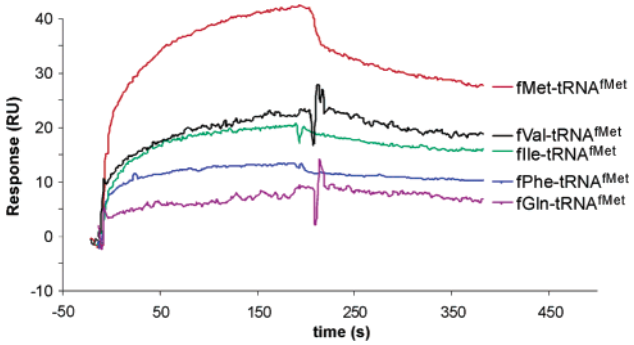

FIGURE 6: Real-time binding of *E. coli* IF2 to fAA-tRNAs. Overlay plot of sensorgrams of 1 μM fMet-tRNA^{fMet}, fVal-tRNA^{fMet}, fIle-tRNA^{fMet}, fPhe-tRNA^{fMet}, fGln-tRNA^{fMet} injected across an IF2-coupled sensor chip surface. The experiments were done in triplicate, including the use of different IF2 surfaces and yielded identical sensorgrams.

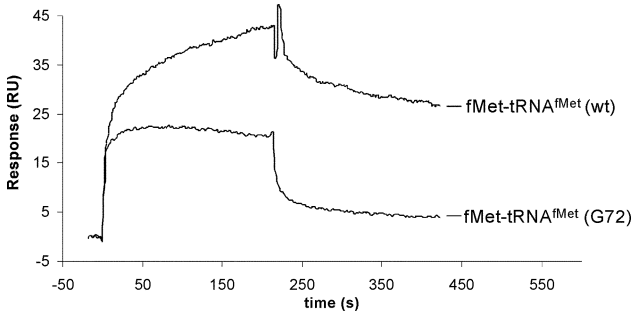

FIGURE 7: Comparative binding of fMet-tRNA^{fMet} and fMet-tRNA^{fMet} (G72) injected across an IF2-coupled sensor chip surface. Typical sensorgrams obtained with 1 μM injections of the tRNAs are shown as overlay plot. The experiments were done in duplicate including the use of different IF2 surfaces.

a simple 1:1 Langmuir interaction model, where A + B $\leftrightarrow$ AB, but we were unable to obtain a satisfactory fit, particularly during the dissociation phase (Figure 5B, inset). Alternative models were used, and it was found that a presumed conformation change after the initial complex formation gave a better fit, characterized by low χ^2 values ($\chi^2 = 0.28$) and random distribution of the residuals (Figure 5B,C). This model invokes that A + B $\leftrightarrow$ AB, which undergoes a conformational change to AB*, that cannot directly dissociate to A + B. The rate constants (k_a and k_d) and the constants describing the conformation change (k_{conf} and k_{-conf}) are given in Table 2. The overall apparent dissociation constant K_D is 0.93 μM and was calculated from the ratio of k_d to k_a multiplied by the ratio of k_{-conf}/k_{conf}. This number is very similar to previous results obtained using different techniques such as protection against chemical deacylation (*30*), gel retardation (*10*), or analytical ultracentrifugation (*31*).

Binding Affinity of the Various fAA-tRNAs to IF2. The importance of the amino acid attached to the mutant initiator tRNAs was tested by injecting fGln-tRNA, fVal-tRNA, fIle-tRNA, and fPhe-tRNA across the IF2 surface. Figure 6 shows sensorgrams obtained with injections of a 1 μM solution of wild-type fMet- and mutant fAA-tRNAs. The mutant tRNAs show slower binding and the following order of binding affinity: fMet-tRNA > fVal-tRNA $\sim$ fIle-tRNA > fPhe-tRNA > fGln-tRNA. Unfortunately, the low binding affinities of the mutants (K_D estimated > 10 μM) prevented an accurate measurement of k_a and k_d values from these sensorgrams.

Role of the C1xA72 Mismatch in the Initiator fMet-tRNA on Binding to IF2. It has long been known that the primary determinant for binding of IF2 is the formyl-group or, more generally, the blocked α-amino group of the amino acid attached to the initiator tRNA (*6*). In previous work and this study, we have established the importance of the amino acid moiety. As a next step we wanted to investigate the role of the C1xA72 mismatch, a unique feature in all eubacterial initiator tRNAs, for IF2 recognition. To this end, we used the G72 mutant initiator tRNA and used SPR to follow its binding to IF2. This tRNA is a very poor substrate for MTF; however, in the presence of MetRS and an excess of MTF, it can be quantitatively aminoacylated and formylated in vitro (*18*). Figure 7 shows the sensorgrams of tRNA injections of a 1 μM solution of the wild-type and G72 mutant fMet-tRNAs. Comparison of the sensorgrams shows that the G72 mutant fMet-tRNA binds poorly to IF2 compared to the wild-type fMet-tRNA. The decrease in the RU values during the later time points (>50 s) of the association curve for the G72 mutant is unusual and could be due to a major conformational change of the complex, rapid dissociation of the complex, or both. The same phenomenon was observed during the binding of the G72 mutant fMet-tRNA at four different concentrations (0.5, 0.7, 1.0, and 1.5 μM) and with two different batches of IF2 immobilized on the CM5 chip (data not shown). Because of this phenomenon, it was not possible to derive an accurate estimate of the dissociation rate constant (k_d) of the IF2·G72 mutant fMet-tRNA complex using the mathematical models available in the Biacore software package. It was possible to obtain an estimate of the association rate constant (k_a) of the IF2·G72 mutant fMet-tRNA complex from the very early time points (5−30 s) of the association curve. The k_a thus derived, which is an average of k_a's obtained from injection of the G72 mutant fMet-tRNA at four different concentrations (0.5−1.5 μM), was 4.16×10^3 M^{-1} s^{-1}. This k_a is about 7-fold lower than that for the IF2·wild-type fMet-tRNA complex (Table 2).

DISCUSSION

The results described in this paper on the effect of overproduction of IleRS, PheRS, MTF, and IF2 on the activity of mutant initiator tRNAs in initiation provide further support to our previous conclusions (i) that a ribosome·initiator tRNA complex can act as an intermediate in

translation initiation and (ii) that IF2 can act as a carrier of fAA-tRNA to the ribosome. The previous conclusions were based on studies of mutant initiator tRNAs aminoacylated with glutamine and valine, and the current work involves mutants aminoacylated with isoleucine and phenylalanine. Analyses of the effect of overproduction of various proteins on the state of aminoacylation and formylation in vivo of the mutant initiator tRNAs and in vitro binding studies of various fAA-tRNAs to IF2 have also provided us with additional information on recognition of the mutant initiator tRNAs by IleRS and PheRS and on the role of amino acids attached to the tRNAs on recognition by MTF and IF2. We discuss below each of these issues.

Recognition of Initiator tRNA by MTF: Role of Amino Acid Attached to the tRNA. The amino acid attached to an initiator tRNA is inspected at least twice prior to its use in initiation of protein synthesis. The first step is in recognition of the amino acid by MTF. There is ample in vitro and in vivo evidence to suggest a hierarchy of preference of the *E. coli* MTF for the identity of the amino acid attached to the initiator tRNA, Met > Gln ~ Phe > Ile ~ Val > Lys. One of the strongest in vivo evidence stems from the observation that a mutant derived from the *E. coli* elongator methionine tRNA that is aminoacylated with lysine, is not formylated, and is, therefore, inactive in initiation. In cells overexpressing MetRS, however, a substantial fraction of the same tRNA is now formylated because it is aminoacylated with methionine and the fMet-tRNA is active in initiation (*15*). The direct analysis of the in vivo formylation status of the initiator tRNA mutants by acid urea gel electrophoresis (with and without MTF overexpression) also enables us to derive an approximate ranking of the effect of the amino acids on formylation of the tRNA (*11*, *13*, *32*) (this work). With mutant initiator tRNAs aminoacylated with glutamine (*13*, *32*) and phenylalanine (this work) very little aminoacylated tRNA is detectable in vivo, the tRNA is either formylated or uncharged, suggesting that these tRNAs are good substrates for MTF. With mutant tRNAs that are aminoacylated with valine and isoleucine (*11*) (this work), MTF must be overproduced to convert all of the aminoacyl-tRNAs to fAA-tRNAs. With the mutant tRNA aminoacylated with lysine, even overexpression of MTF does not lead to any significant formylation of the tRNA (*15*).

Kinetic measurements of in vitro formylation reactions of initiator tRNAs complement these observations, in that k_{cat}/K_M of initiator tRNA aminoacylated with different amino acids follows the order Met > Phe > Val (*28*) and Met > Ile ~ Val (*33*). The structural reason for the preference or tolerance of MTF for certain amino acids lies in the nature of the binding cavity for methionine near the active site of the enzyme. The crystal structure of *E. coli* MTF·fMet-tRNA complex shows that the methionine binding cavity consists of a cluster of hydrophobic side chains and holds the methionine moiety of the Met-tRNA substrate in place but does not make any specific contacts with it, so that some alternate amino acids are permitted (*34*). This preference of *E. coli* MTF for methionine is even more pronounced in the bovine mitochondrial MTF. For this enzyme, the amino acid is the most important identity element on the initiator tRNA (*33*), whereas for *E. coli* MTF, it is the presence of a C1xA72 mismatch at the end of the acceptor stem (*5*, *32*).

Recognition of Initiator tRNA by IF2: Role of Amino Acid Attached to the tRNA and Role of the Unique C1xA72 Mismatch. The second inspection of the amino acid during initiation of protein synthesis occurs during recognition of the initiator fAA-tRNA by IF2. The SPR results (Figure 6) and the results of gel shift analyses (*10*) indicate that as for MTF, there is a hierarchy of preferred amino acids for IF2: fMet > fVal > fIle > fPhe > fGln. A first indication that fGln-tRNA is a poor substrate for IF2 came from the observation that overproduction of IF2 led to a large increase in CAT activity in cells expressing the mutant tRNA which carries fGln (*9*). In contrast, there was no such effect in cells expressing a mutant initiator tRNA carrying fVal (*11*). Here, we have also shown that overproduction of IF2 leads to moderate increases in CAT activity of mutant tRNAs carrying fIle and fPhe. Thus, there is a reciprocal relationship between the effect of overproduction of IF2 on the activity of mutant tRNAs in initiation and the binding affinity of the various fAA-tRNAs to IF2 (Table 1 and Figure 6) (*11*). Taking the results of these in vitro and in vivo studies together, we conclude that IF2 binding follows the order: fMet > fVal > fIle > fPhe > fGln. It is possible that the very poor binding of fGln-tRNA is because the amino acid binding pocket prefers hydrophobic side chains (*6*).

There is no conclusive structural information yet on how IF2 recognizes fMet-tRNA. The fMet moiety of the fMet-tRNA has long been thought to be the primary determinant for binding to IF2. It has been suggested that a 90 amino acid C-terminal subdomain (domain IV) of *B. stearothermophilus* IF2 and a similar domain of *Thermus thermophilus* IF2 (*35*) has all of the fMet-tRNA binding site (*36*). The NMR structure of this domain of *B. stearothermophilus* IF2 has been determined and the CCA-fMet end of the tRNA has been modeled into the structure (*37*). It has also been suggested that the CAACCA-fMet portion of the tRNA is all that is needed for binding to IF2 (*38*), although a previous study suggested that the interaction extended to the minor groove of the TΨC stem and loop of the tRNA (*39*). NMR analysis of the 90 amino acid fragment of *B. stearothermophilus* IF2 in the presence of fMet or methionine indicated a specific, although weak, interaction with fMet, but not methionine (*38*). Studies with the C-terminal domain of *T. thermophilus* IF2 have shown that the fMet-AMP is an effective competitor for binding of fMet-tRNA (*35*).

The molecular basis for the poor binding of the G72 mutant fMet-tRNA to IF2 (Figure 7) is unknown. IF2 could interact directly with the A72 that was mutated to G72. Alternatively, IF2, like MTF, might be sensitive to the presence of a strong Watson Crick base pair between positions 1 and 72 at the end of the acceptor stem (*34*, *40*, *41*). Further studies are necessary to decide among these possibilities. The presence of a strong base pair at the end of the acceptor stem could make the CCA end of the tRNA too rigid to fit well into the tRNA binding surface of IF2. Alternatively, it could affect the spatial relationship of the acceptor stem−TΨC stem backbone with respect to the fMet attached to the 3′-end of the tRNA. Overall, it is interesting that IF2 seems to take advantage of several of the characteristic features in the eubacterial initiator fMet-tRNA—the amino acid methionine, the formyl-group, and the C1xA72 mismatch—to selectively bind to it.

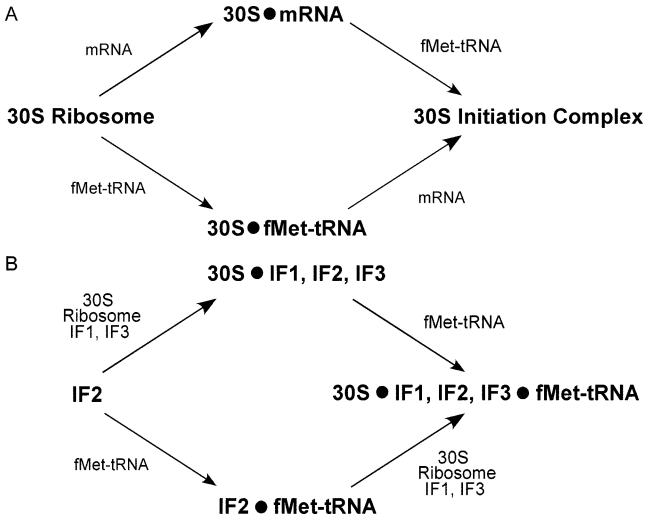

FIGURE 8: Schematic representation of possible pathways in initiation of translation illustrating the hypothetical intermediates for assembly of the (A) 30S:mRNA:fMet-tRNA and (B) 30S:IF1/IF2/IF3:fMet-tRNA complexes.

The conformational change of IF2·wild-type fMet-tRNA complex that we have inferred from the Biacore experiments clearly needs further confirmation based on biochemical data. However, there are several lines of indirect evidence in support of it. (i) There have been suggestions of a long-range structural change around the GTP/GDP switch region of the IF2 molecule triggered by tRNA binding based on protection of IF2 of *B. stearothermophilus* against proteolysis (*42*). (ii) A structural change of IF2 has also been invoked to explain unexpected results from a cross-linking study performed with IF2 and the initiator fMet-tRNA from *E. coli* (*43*). (iii) The X-ray structure of a related initiation factor, eIF5B, from *Methanococcus thermolithotrophicus* has been solved. This protein exploits an articulated lever mechanism to transduce the energy of GTP binding and a concomitant small structural change in the switch 2 region from the active site on the G domain to the C-terminus, which covers a distance of more than 90 Å (*44*).

Ribosome·fMet-tRNA Complex as an Intermediate in the Initiation of Protein Synthesis. We have shown that mutant CAT genes with UAG, AUC, GUC, UUC, and CAG initiation codons produce considerably more CAT protein in the presence of the appropriate mutant initiator tRNA than the CAT gene with the wild-type initiation codon (Table 1). The initiation codons used include all four nucleotides at position +1 and two different nucleotides each at positions 2 and 3; the amino acids used to initiate protein synthesis include fGln, fVal, fIle, and fPhe. Thus, the increased production of CAT protein in cells carrying a combination of mutant CAT gene and mutant initiator tRNA appears to be a general phenomenon. As pointed out before (*9*, *11*), this is most likely due to the fact that the 30S ribosome·fAA-tRNA complex is an intermediate in initiation of translation in these cases (Figure 8A). In cells overproducing the mutant initiator tRNAs, a significant fraction of the ribosomes would be loaded with the mutant fAA-tRNA and these ribosomes would preferentially select the mutant CAT mRNA for translation via codon/anticodon recognition (*4*) over other mRNAs. In contrast, the wild-type CAT mRNA with a normal AUG start codon would be translated like the bulk of *E. coli* mRNAs. Recently, working with leaderless mRNAs, Blasi, Gualerzi, and co-workers (*45*) have also concluded that a ribosome·fMet-tRNA complex is an intermediate in translation initiation in *E. coli*.

IF2 as a Possible Carrier of fAA-tRNAs to the Ribosome. In general, tRNAs are carried to the ribosome by a protein carrier: aminoacyl-tRNAs by elongation factor EF-Tu or EF-1 (*2*), the selenocysteinyl-tRNA by the SELB protein (*46*), and eukaryotic initiator Met-tRNAs by eIF2 (*47*). How the *E. coli* fMet-tRNA gets to the ribosome and whether IF2 acts as a carrier of the tRNA is, however, not established. It has been suggested that IF2 is usually already bound to the 30S subunit and selects the fMet-tRNA from the pool of cellular tRNAs as part of the 30S initiation complex (*48*). Support for this model is derived from the estimated concentrations of IF2 in cells (approximately 4 μM), and the finding that the apparent dissociation constant of the 30S ribosomal subunit·IF2 complex is in the high nanomolar range (*49*). Furthermore, the IF2·fMet-tRNA binary complex is somewhat weak in vitro and is readily dissociated by physiological concentrations of magnesium and monovalent cations (*6*), although it is stable to glycerol gradient centrifugation (*50*).

Results obtained in this and previous studies (*9*, *10*) are, however, hard to explain, if the ribosome-bound IF2 proteins are the only biologically active population of the initiation factor in cells. If 30S ribosomes are already saturated with IF2, overproduction of IF2 should not be able to compensate for reduced affinity of initiator tRNA mutants aminoacylated with glutamine, isoleucine, or phenylalanine. Our results are easier to understand if IF2 can act as a carrier of fAA-tRNAs to the ribosome. There is a good reciprocal correlation between the effect of overproduction of IF2 on the activity of initiator fMet-, fVal-, fIle-, fPhe-, and fGln-tRNAs in initiation in *E. coli* and their affinities for IF2 in vitro (see previous section). This indicates that, at least under conditions of IF2 overproduction, formation of an IF2·fAA-tRNA binary complex is possible. This would explain the increased activity of fGln-, fIle-, and fPhe-tRNAs in initiation when IF2 is overproduced, by increased binding of these tRNAs to IF2 leading to their increased utilization in initiation. It might also be speculated that, if a binary complex is stable enough in vivo, it could occur even under circumstances of normal IF2 concentrations, perhaps as an alternative pathway (Figure 8B). A similar conclusion has been reached by Blanquet, Grunberg-Manago, and co-workers (*51*) during their studies on the effect of overproduction of IF2 on utilization of unformylated initiator tRNA in initiation in *E. coli*. An alternative hypothesis is that the increased activity of some of the mutant tRNAs in initiation in cells overproducing IF2 is due to the excess of IF2 forming a binary complex with the wild-type fMet-tRNA, for which it has the highest affinity (*52*). This would lead to reduction in levels of free wild-type fMet-tRNA compared to mutant fAA-tRNAs, resulting in the preferential selection and utilization of mutant fAA-tRNAs by IF2 bound to 30S ribosome. Such a hypothesis, however, would not explain how the fAA-tRNA gets to the ribosome. In this regard, it is particularly interesting to note that while the other *E. coli* initiation factors IF1 and IF3 bind to the 30S ribosome and protect specific bases of the 16S rRNA from reacting with chemical probes, IF2 does not protect any specific bases in 16S rRNA (*53*), although it does so in 23S rRNA (*54*). This suggests either that IF2

does not bind stably to the 30S ribosome without the initiator fMet-tRNA or that its interactions with the 30S ribosome involve primarily the ribosomal protein(s) and not rRNA.

Requirements for Optimal Activity of Mutant tRNAs in Initiation. Finally, we have identified the limiting factors in initiation of protein synthesis using mutant initiator tRNAs aminoacylated with amino acids other than methionine. For each mutant initiator tRNA, the requirements for its maximal utilization are different. Optimal synthesis of CAT protein using the U35A36 mutant tRNA depends mainly on overproduction of IF2, whereas that using the G34C36 mutant, depends on overproduction of ValRS and MTF. For the G34 mutant all three proteins, IleRS, MTF, and IF2 need to be overproduced, whereas for the G34A36 mutant, only PheRS and IF2 need to be overproduced. The general conclusion, however, is that there is no apparent restriction against initiation by any amino acid or by any particular initiation codon. This is in contrast to previous indications that initiation with fPhe might be particularly inefficient (*12*). Thus, it appears that if complete aminoacylation and formylation of the mutant initiator tRNAs and efficient delivery of the fAA-tRNAs to the ribosome are assured (by overproducing the aaRS, MTF and IF2 as needed), more of the CAT reporter protein is made irrespective of the amino acid utilized for initiation. These findings reinforce the prospect of overproducing proteins carrying different amino acids at their N-termini using mutant initiator tRNAs.

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