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Initiation of in Vivo Protein Synthesis with Non-Methionine Amino Acids†

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ABSTRACT: Methionine is the universal amino acid for initiation of protein synthesis in all known organisms. The amino acid is coupled to a specific initiator methionine tRNA by methionyl-tRNA synthetase. In *Escherichia coli*, attachment of methionine to the initiator tRNA ($tRNA^{fMet}$) has been shown to be dependent on synthetase recognition of the methionine anticodon CAU (complementary to the initiation codon AUG), [Schulman, L. H., & Pelka, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6755–6759]. We show here that alteration of the anticodon of $tRNA^{fMet}$ to GAC or GAA leads to aminoacylation of the initiator tRNA with valine or phenylalanine. In addition, $tRNA^{fMet}$ carrying these amino acids initiates in vivo protein synthesis when provided with initiation codons complementary to the modified anticodons. These results indicate that the sequence of the anticodon of $tRNA^{fMet}$ dictates the identity of the amino acid attached to the initiator tRNA in vivo and that there are no subsequent steps which prevent initiation of *E. coli* protein synthesis by valine and phenylalanine. The methods described here also provide a convenient in vivo assay for further examination of the role of the anticodon in tRNA amino acid acceptor identity.

Methionine is the only naturally occurring amino acid known to initiate protein synthesis in vivo (Lucas-Lenard & Lipmann, 1971). In prokaryotes, the amino acid is first attached to the initiator methionine tRNA by methionyl-tRNA synthetase and subsequently formylated by methionyl-tRNA transformylase. The resulting fMet-$tRNA^{fMet}$ [1] initiates protein synthesis in a reaction dependent on initiation factor 2 (IF-2). In vitro studies indicate that methionine or methionine analogues are strictly required for the aminoacylation reaction but that other naturally occurring amino acids can substitute for methionine in the formylation and initiation steps of protein synthesis (Lucas-Lenard & Lipmann, 1971; Giege et al., 1973a,b; Sundari et al., 1976; Leon et al., 1979). This raises the possibility that non-methionine amino acids, if coupled to the initiator tRNA, could initiate polypeptide synthesis in vivo.

Aminoacylation of *Escherichia coli* methionine tRNAs depends on recognition of the methionine anticodon CAU by *E. coli* methionyl-tRNA synthetase (Schulman & Pelka, 1983). Mutations in this sequence lead to loss of methionine acceptor activity and, in some cases, to acquisition of a new amino acid acceptor activity corresponding to that of the altered anticodon sequence (Schulman & Pelka, 1985, 1988, 1989, 1990). In order to determine whether in vivo aminoacylation of $tRNA^{fMet}$ and initiation of protein synthesis can occur with non-methionine amino acids, we have constructed plasmid-borne *lacZ* derivatives containing non-methionine initiation codons. We show here that β-galactosidase synthesis from these plasmids is initiated with non-methionine amino acids when cells are complemented with mutant initiator tRNAs containing the corresponding anticodon sequences, as schematically illustrated in Figure 1.

Experimental Procedures

Materials

E. coli $tRNA^{fMet}$ having a specific activity of 1700 pmol/A_{260} unit was purchased from Boehringer. *E. coli*

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[1] Abbreviations: $tRNA^{fMet}$, *E. coli* initiator methionine tRNA; $tRNA^{fMet}$(GAC), $tRNA^{fMet}$ containing the valine anticodon GAC; $tRNA^{fMet}$(GAA), $tRNA^{fMet}$ containing the phenylalanine anticodon GAA; $tRNA^{Val}$(U*AC), major *E. coli* valine tRNA having the anticodon U*AC, where U* is 5-(carboxymethoxy)uridine; $tRNA^{Phe}$(GAA), *E. coli* phenylalanine tRNA; IAA, 3β-indoleacrylic acid; TCA, trichloroacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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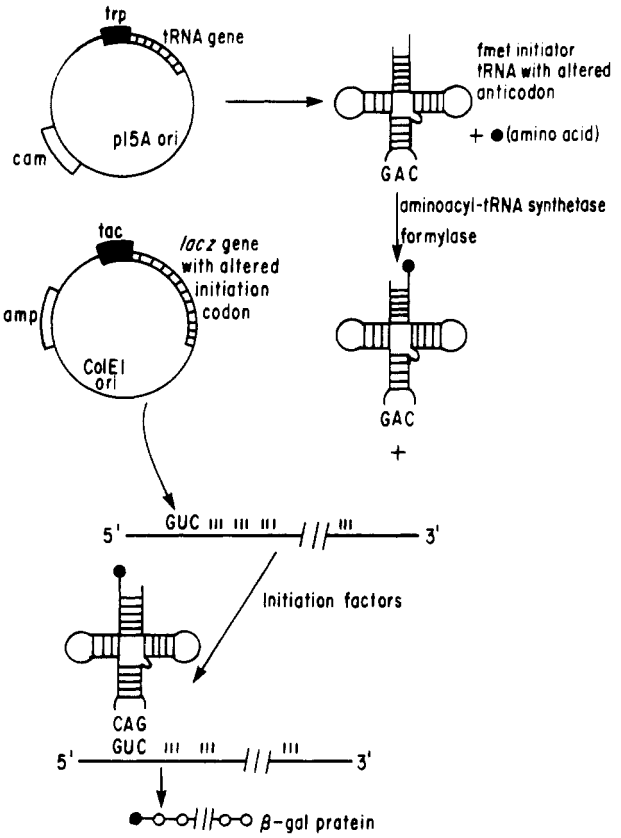

FIGURE 1: Schematic illustration of the initiation of β-galactosidase synthesis from a valine initiation codon by $tRNA^{fMet}$ having a complementary valine anticodon sequence.

$tRNA^{Val}$ having a specific activity of 1100 pmol/A_{260} unit and *E. coli* $tRNA^{Phe}$ having a specific activity of 1300 pmol/A_{260} unit were obtained from Subriden RNA. *E. coli* methionyl-tRNA synthetase and *E. coli* valyl-tRNA synthetase were purified as described before (Schulman & Pelka, 1988). Purified *E. coli* phenylalanyl-tRNA synthetase was a generous gift from E. Holler. T4 RNA ligase was purified from *E. coli* infected with T4 phage strain SP62, am N82 (Higgins et al., 1977). [^{35}S]Methionine, [^{14}C]valine, and [^{14}C]phenylalanine were purchased from Amersham. Deoxyoligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. Enzymes for plasmid constructions were obtained from New England Biolabs. Trinucleotides pGACp and pGAAp were synthesized as described before (Schulman et al., 1983). IAA was obtained from Sigma.

Methods

In Vitro Synthesis of $tRNA^{fMet}$ Anticodon Mutants. Half-molecule-sized fragments of $tRNA^{fMet}$ missing the anticodon nucleotides and two nucleotides of the 3′-terminal CpCpA sequence were isolated after limited digestion of the tRNA with pancreatic RNase (Schulman et al., 1983). Intact molecules containing GAC and GAA anticodon sequences were synthesized by joining trinucleotides to the half-molecules by use of T4 RNA ligase and polynucleotide kinase, and the 3′-terminal sequence was enzymatically replaced by use of tRNA nucleotidyltransferase, as described before (Schulman et al., 1983).

Aminoacylation Assays. Methionine and valine acceptor activities of $tRNA^{fMet}$ derivatives were measured as described elsewhere (Schulman & Pelka, 1988). Reaction mixtures for assay of phenylalanine acceptance contained 100 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 2 mM reduced glutathione, 2 mM ATP, 20 μM [^{14}C]phenylalanine (620 cpm/pmol), 0.2 mM L-tyrosine, 100 μg/mL bovine serum albumin, 1% glycerol, 2 μM tRNA, and 80 units/mL phenylalanyl-tRNA synthetase. Incubations were at 28 °C for various times, and reactions were stopped by pipetting of aliquots (5 μL) into 2 mL of cold 10% TCA containing 1 mg/mL DL-phenylalanine. Samples were then passed through Whatman GF/C filters and the filters washed three times with 5-mL portions of cold 5% TCA containing 1 mg/mL DL-phenylalanine and then with 5 mL of 70% ethanol. Filters were dried and placed in 5 mL of Econofluor (New England Nuclear), and the radioactivity was measured in a liquid scintillation counter.

Plasmid Constructions. A synthetic *trp* promoter, $tRNA^{fMet}$ gene, and *rrnC* transcription terminator were constructed from overlapping deoxynucleotides (Normanly et al., 1986a,b) and inserted between the *Sal*I and *Hin*dIII restriction enzyme sites of a pACYC184 plasmid containing a p15A origin of replication and a chloramphenicol-resistance gene (Chang & Cohen, 1978) to yield plasmid pHP12. Derivatives of the initiator tRNA with altered anticodon sequences were prepared in the same way and the structures confirmed by DNA sequencing.

A pBR322 plasmid containing a pseudo-wild-type *lacZ* gene was obtained from R. Weiss (Weiss et al., 1987). A *tac* promoter was inserted just upstream of the *Eco*RI site to yield plasmid pBW5. The initiation codon was changed by ligating pairs of annealed 27-mer deoxynucleotides between the unique *Eco*RI and *Hin*dIII sites of the vector.

Assay, Purification, and Sequencing of β-Galactosidase. *E. coli* JM109 cells (*recA1* Δ *lac-pro endA1 gyrA96 thi-1 hsdr17 supE44 relA1*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}*Z* Δ *M15*; Yanisch-Perron et al., 1985) freshly transformed with *lacZ* constructs were grown overnight from single colonies in minimal M9 medium supplemented with 0.4% casamino acids and 10 μg/mL vitamin B_1 and containing 100 μg/mL ampicillin and 2 μg/mL IAA. Overnight cultures were diluted 1:100 into fresh medium, IPTG was added to 2 mM after 1 h, and the cells were grown to stationary phase. β-Galactosidase assays were carried out as described by Miller (1972). Cells containing both pBW5 and pHP12 plasmids were grown in the same way except that the medium also contained 30 μg/mL chloramphenicol.

E. coli strain MY649 (Δ *lac-pro ara thi recA56* F$^-$; Curran & Yarus, 1986) was used for growth of large-scale cultures for purification of β-galactosidase. Cells were grown as described above except that the medium was supplemented with 0.4% glucose and 50 μg/mL proline. In addition, no IPTG was added to the medium. Cells were pelleted by centrifugation and resuspended in 100 mM Tris-HCl, pH 7.4, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride. The cells were disrupted by sonication and centrifuged (10000*g*) to remove cell debris. β-Galactosidase was purified from the soluble crude cell extract on an antibody affinity column (Promega) according to the manufacturer's protocol. The yield of protein was determined with the Bio-Rad assay, and the purified protein was subjected to four cycles of Edman degradation on a gas-phase protein sequencer. No deblocking procedure was used to remove potential formyl groups from the N-terminus of the protein.

Determination of tRNA Levels. Levels of $tRNA^{fMet}$ derivatives in cells carrying pHP12 plasmids were determined by Northern hybridization of a $tRNA^{fMet}$-specific deoxynucleotide probe to filter-bound tRNA. Cells were grown as described above, and crude tRNA was isolated according to the procedure of Smith and Yarus (1989). Two micrograms of RNA per lane was loaded onto a 10% polyacrylamide gel

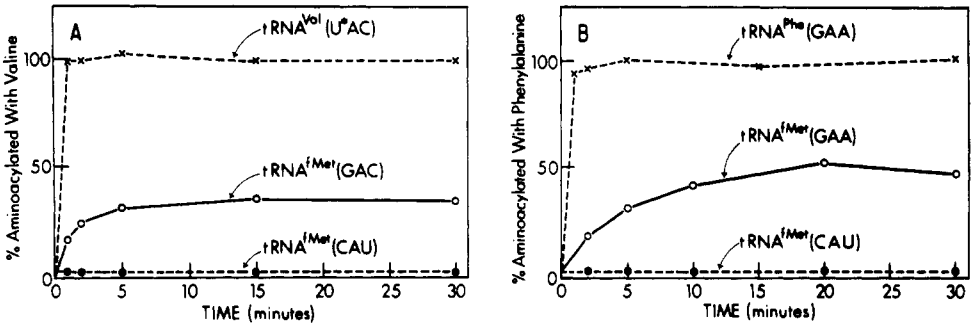

FIGURE 2: In vitro aminoacylation of $tRNA^{fMet}$ anticodon derivatives with valine and phenylalanine. Anticodon derivatives of $tRNA^{fMet}$ were synthesized by anticodon replacement using bacteriophage T4 RNA ligase and characterized as described before (Schulman et al., 1983). Aminoacylation assays were carried out as described under Experimental Procedures. (Panel A) tRNA concentrations were 1 μM, and assays contained 153 units/mL *E. coli* valyl-tRNA synthetase. (Panel B) tRNA concentrations were 2 μM, and assays contained 80 units/mL *E. coli* phenylalanyl-tRNA synthetase.

containing 7 M urea and electrophoresed in parallel with standards (10–100 ng) of pure $tRNA^{fMet}$ for 10 h at 4 °C. The gels were washed with 40 mM Tris–acetate and 1 mM EDTA for 10 min at room temperature, and then the RNA was transferred to Amersham Hybond-N hybridization filters by electroelution at 300 mA for 3 h at 4 °C. The RNA was cross-linked to the filters with a Stratalinker. The filters were washed in 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7, and 0.01% sodium dodecyl sulfate for 1 h at 65 °C. Prehybridization, hybridization with 50–75 pmol of 5′ ^{32}P-labeled probe, and quantitation of tRNA were carried out as described by Smith and Yarus (1989). The deoxyoligonucleotide probe used was complementary to residues 48–71 of the $tRNA^{fMet}$ sequence, a region that does not include the anticodon sequence.

Results

Synthesis and Aminoacylation of $tRNA^{fMet}$ Derivatives Containing Altered Anticodons. Derivatives of $tRNA^{fMet}$ containing GAC (valine) and GAA (phenylalanine) anticodons were synthesized according to a procedure similar to that originally described by Bruce and Uhlenbeck (1982). Limited digestion of $tRNA^{fMet}$ with pancreatic RNase was used to generate half-molecule-sized fragments missing the anticodon nucleotides and the two terminal nucleotides of the 3′ CCA sequence. The dephosphorylated 5′ half-molecule was annealed with the 3′ half-molecule containing a 3′-phosphate group. The trinucleotides pGACp and pGAAp were joined to the 3′-OH group of the 5′ fragment by use of T4 RNA ligase. The extended 5′ fragment and the 3′ fragment were dephosphorylated at the 3′ termini and phosphorylated at the 5′ termini with polynucleotide kinase and ATP. The anticodon loop was joined by incubation of the annealed complex with RNA ligase, and the 3′-terminal CCA sequence was enzymatically repaired with tRNA nucleotidyltransferase, CTP, and ATP. The final products were isolated by polyacrylamide gel electrophoresis in 7 M urea and sequenced to confirm the structure (Schulman & Pelka, 1983).

Assays of the $tRNA^{fMet}$ derivatives with excess methionyl-tRNA synthetase yielded no detectable methionine acceptor activity, indicating that the mutant initiator tRNAs were no longer substrates for the cognate synthetase (data not shown). Substitution of the methionine anticodon CAU with the valine anticodon GAC resulted in significant aminoacylation of $tRNA^{fMet}$ with valine by valyl-tRNA synthetase under conditions where wild-type $tRNA^{fMet}$ showed no activity (Figure 2A). Similarly, $tRNA^{fMet}$ containing the phenylalanine anticodon GAA showed greatly enhanced phenylalanine acceptor activity when assayed with phenylalanyl-tRNA synthetase (Figure 2B). Both of the modified initiator tRNAs exhibited incomplete charging with the non-methionine amino acids under conditions leading to rapid and quantitative aminoacylation of wild-type $tRNA^{Val}$ and $tRNA^{Phe}$ (Figure 2).

In Vivo Synthesis of β-Galactosidase from Non-Methionine Initiation Codons. In order to study the in vivo aminoacylation and initiation of protein synthesis by $tRNA^{fMet}$ anticodon derivatives, synthetic tRNA genes containg sequences corresponding to wild-type $tRNA^{fMet}$(CAU) and to the anticodon derivatives $tRNA^{fMet}$(GAC) and $tRNA^{fMet}$(GAA) were constructed in a pACYC184 plasmid containing a *trp* promoter, *rrnC* terminator, p15A origin of replication, and chloramphenicol-resistance gene. Expression of the tRNA from the *trp* promoter was induced in *trpR*$^+$ strains by reducing the endogenous level of tryptophan by addition of the tryptophan analogue β-indoleacrylic acid (IAA) to the medium (Nichols & Yanofsky, 1983). Northern hybridization analyses revealed that overnight cultures grown in the presence of 2 μg/mL IAA as described under Experimental Procedures produced a level of $tRNA^{fMet}$ from these plasmids that was 1–2 times the level of endogenous $tRNA^{fMet}$. A compatible pBR322 plasmid containing a *tac* promoter and pseudo-wild-type *lacZ* gene was used for expression of β-galactosidase in host cells containing the tRNA expression vector. The sequence of the initiation region of the gene containing a methionine start codon is shown in Figure 3. Following codon 11, the DNA encodes the wild-type β-galactosidase sequence beginning with Ser7 of the protein. The sequence of the start codon was changed to GUC (valine) and UUC (phenylalanine) by use of the conveniently located unique *Eco*RI and *Hin*dIII restriction enzyme sites of the vector.

The relative efficiency of β-galactosidase synthesis from the different plasmid-borne *lacZ* genes was determined by expression of the proteins in a *lacI*^{Q} host on induction with IPTG. Synthesis from *lacZ* containing the AUG initiation codon [*lacZ*(AUG)] was highly efficient, yielding over 100 000 units (Miller, 1972) of β-galactosidase in overnight cultures when endogenous $tRNA^{fMet}$ served as the sole source of initiator tRNA. Initiation from *lacZ*(GUC) and *lacZ*(UUC) under the same conditions produced only 1% and 0.4% of this level, respectively (Table I).

Synthesis of β-galactosidase from the non-methionine initiation codons was significantly increased when cells were grown in the presence of $tRNA^{fMet}$ containing the complementary anticodon (Table I). Levels of enzyme activity were 7-fold higher in cultures containing both $tRNA^{fMet}$(GAC) and *lacZ*(GUC), while activity increased 5-fold when $tRNA^{fMet}$(GAA) was expressed in cells harboring *lacZ*(UUC).

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EcoRI          Start HindIII
GAATTCGTTCTTAGGGGGTATCATGAAAAGCTTAGCTGTAATTAGCGGCCCTAAT....
                    MetLysSerLeuAlaValIleSerGlyProAsn....
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FIGURE 3: DNA and protein sequence of the initiation region of the pseudo-wild-type β-galactosidase gene.

Table I: Yield of β-Galactosidase from *lacZ* Genes Containing Methionine and Non-Methionine Initiation Codons[a]

initiation codon	complementary $tRNA^{fMet}$ added	units of β-galactosidase
AUG	+	105 640 ± 2 210
	−	104 295 ± 7 465
GUC	+	8 665 ± 655
	−	1 235 ± 120
UUC	+	2 165 ± 325
	−	410 ± 30

[a] *E. coli* JM109 cells harboring plasmids containing *lacZ* genes with different initiation codons were grown in the presence and absence of plasmids carrying initiator tRNAs with complementary anticodon sequences, as described under Experimental Procedures. Yields of β-galactosidase were determined as described by Miller (1972) and represent the average of four separate determinations.

Expression of wild-type $tRNA^{fMet}$ from pHP12 in the presence of *lacZ*(AUG) resulted in no increase in the level of β-galactosidase over that observed in the absence of the plasmid-borne tRNA.

N-Terminal Sequence of β-Galactosidase Derivatives. The identity of the amino acid used to initiate β-galactosidase synthesis from each *lacZ* gene was determined by protein sequencing (Table II). β-Galactosidase was purified on an antibody affinity column (Promega) and subjected to four cycles of Edman degradation on a gas-phase protein sequencer. Protein initiated by endogenous wild-type $tRNA^{fMet}$ from *lacZ* containing the AUG initiation codon had only methionine at the N-terminus. Only traces of lysine (0–0.2%) were found in the first sequencing cycle, indicating that the protein is resistant to the action of the aminopeptidase which removes N-terminal methionine from many *E. coli* proteins (Lucas-Lenard & Lipmann, 1971). Methionine was also the N-terminal amino acid inserted in response to the GUC initiation codon in the absence of the $tRNA^{fMet}$ derivative containing the complementary valine anticodon; however, a mixture of methionine and phenylalanine was found at the N-terminus of protein synthesized from *lacZ*(UUC) in the absence of the complementary $tRNA^{fMet}$(GAA) in several independent experiments. Expression of $tRNA^{fMet}$(GAC) in cells harboring the *lacZ*(GUC) gene led to synthesis of β-galactosidase having N-terminal valine, while an increase in the relative amount of phenylalanine was found at the N-terminus of protein initiated from the UUC start codon in the presence of $tRNA^{fMet}$(GAA).

Discussion

All amino acids function efficiently in the elongation steps of protein synthesis; however, methionine is the only amino acid used for initiation. The present study shows that the major factor in the selection of the initiating amino acid in vivo is the amino acid acceptor identity of the initiator tRNA. Initiation of *E. coli* protein synthesis by valine and phenylalanine reveals that none of the steps subsequent to aminoacylation of $tRNA^{fMet}$ has a strict requirement for the universal initiating amino acid, methionine. The efficiency of initiation by $tRNA^{fMet}$ carrying non-methionine amino acids is lower than that observed with similar levels of wild-type initiator tRNA, however (Table I). The present in vitro mischarging studies show that $tRNA^{fMet}$(GAC) and $tRNA^{fMet}$(GAA) are less efficiently aminoacylated by valyl- and phenylalanyl-tRNA synthetases than wild-type cognate tRNAs (Figure 2). In addition, previous studies have shown that both *E. coli* transformylase and initiation factor 2 prefer methionine to non-methionine amino acids (Giege et al., 1973a,b; Sundari et al., 1976); thus, a combination of reduced efficiency in several steps in the initiation pathway is likely to contribute to the lower level of protein synthesized in the presence of the mutant initiator tRNAs. Little is known about the amino acid requirement for initiation of protein synthesis in eukaryotic systems; however, in vitro experiments have indicated that eukaryotic IF-2 strongly discriminates against the mammalian initiator tRNA when it is mischarged with isoleucine (Wagner et al., 1984), suggesting that higher organisms may have evolved in a manner that more strictly prohibits the use of non-methionine amino acids in the initiation pathway.

AUG, GUG, UUG, and AUU have all been found at the translation start site of wild-type *E. coli* genes, although much higher levels of expression are observed for proteins initiated from the common AUG codon (Kozak, 1983; Butler et al., 1987). The GUC and UUC initiation codons used in this study are related to GUG and UUG by a single base change and are found to produce a low level of β-galactosidase in the absence of the complementary initiator tRNAs (Table I). Reports of weak initiation activity from a variety of non-methionine codons in both prokaryotic and eukaryotic organisms have appeared previously (Kozak, 1983; Zitomer et al., 1984; Hann et al., 1988; Cigan et al., 1988; Peabody, 1989); however, the identity of the initiating amino acid has not usually been directly determined. In this study, initiation from the GUC codon was found to occur exclusively with methionine, indicating a low level of misreading of the non-methionine initiation codon by endogenous $tRNA^{fMet}$. Still weaker initiation occurred from the UUC initiation codon in the absence of the complementary $tRNA^{fMet}$(GAA); however, in this case, a portion of the protein had phenylalanine at the

Table II: Amino Acid Sequence of the N-Terminus of β-Galactosidase Synthesized from Genes Containing Methionine and Non-Methionine Initiation Codons in the Presence and Absence of Complementary Initiator tRNAs[a]

initiation sequence		AUG-AAA-AGC-UUA... Met-Lys-Ser-Leu		GUC-AAA-AGC-UUA... Val-Lys-Ser-Leu				UUC-AAA-AGC-UUA... Phe-Lys-Ser-Leu			
initiator tRNA added		none		none		$tRNA^{fMet}$(GAC)		none		$tRNA^{fMet}$ (GAA)	
pmol of protein		456		348		253		134		111	
cycle	amino acid	pmol	% yield	pmol	% yield	pmol	% yield	pmol	% yield	pmol	% yield
1	Met	228	50	169	49	17	7	50	37	9	8
	Val	0	0	3	1	120	47	1	<1	3	3
	Phe	3	<1	3	<1	3	1	15	11	37	33
2	Lys	160	35	107	37	73	29	40	30	33	30
3	Ser	30	7	35	10	19	8	12	9	12	11
4	Leu	250	55	175	50	97	38	84	63	68	61

[a] *E. coli* MY649 cells harboring plasmids containing *lacZ* genes ± plasmids carrying initiator tRNAs with anticodons complementary to each initiator codon were grown to stationary phase, and proteins were purified and sequenced as described under Experimental Procedures. The reported yields of serine have not been corrected for conversion to dehydroalanine during the Edman degradation.

N-terminus (Table II). Initiation with phenylalanine was approximately one-third as efficient as initiation with methionine from the UUC codon, or about 0.1% of the level of initiation from *lacZ*(AUG) by wild-type $tRNA^{fMet}$. We suggest that this highly inefficient initiation reaction utilizes endogenous Phe-$tRNA^{Phe}$. This explanation seems much more likely than the alternative proposal that endogenous wild-type $tRNA^{fMet}$ is mischarged with phenylalanine in vivo and misreads UUC. *N*-Acetyl-Phe-$tRNA^{Phe}$ has been widely used for in vitro studies of initiation from poly(U), and Phe-$tRNA^{Phe}$ has been shown to efficiently initiate in vitro polyphenylalanine synthesis at elevated Mg^{2+} concentrations (Lucas-Lenard & Lipmann, 1971), indicating that $tRNA^{Phe}$ can mimick the activity of $tRNA^{fMet}$ under certain conditions. It is expected that such activity would not normally be seen in vivo since UUU and UUC codons would rarely occur in an mRNA context favorable for initiation of protein synthesis.

Complementation of *lacZ*(GUC) with $tRNA^{fMet}$(GAC) and of *lacZ*(UUC) with $tRNA^{fMet}$(GAA) leads to increased synthesis of protein from the non-methionine initiation codons (Table I), and this increase is seen to result in a corresponding increase in valine and phenylalanine at the N-terminus of β-galactosidase (Table II). Complementation of *lacZ*(AUG) with plasmid-borne wild-type $tRNA^{fMet}$ produces no increase in synthesis of β-galactosidase from the AUG codon (Table I), indicating that the level of endogenous initiator tRNA is sufficient to support maximal expression of protein from the methionine initiation codon.

In addition to the novelty of generating *E. coli* proteins initiated with non-methionine amino acids, our experiments have also demonstrated that the anticodon sequence of $tRNA^{fMet}$ determines the amino acid that will be inserted into protein by the tRNA in vivo. This confirms our earlier in vitro studies on the role of the anticodon in aminoacylation of *E. coli* methionine and valine tRNAs (Schulman & Pelka, 1983, 1988) and further indicates the importance of the anticodon for in vivo tRNA recognition by *E. coli* phenylalanyl-tRNA synthetase. Studies on both the yeast and *E. coli* phenylalanine enzymes by Uhlenbeck and co-workers have shown that the anticodon sequence is an important element in determining the phenylalanine acceptor activity of tRNAs in vitro (Bruce & Uhlenbeck, 1982; Tinkle and Uhlenbeck, personal communication). Sites outside of the anticodon have previously been shown to contribute to *E. coli* $tRNA^{Phe}$ identity in vivo (Normanly et al., 1986b; McClain & Foss, 1988). Other in vitro and in vivo studies indicate that many *E. coli* aminoacyl-tRNA synthetases require one or more specific anticodon bases for accurate and efficient aminoacylation of tRNA substrates (Kisselev, 1985; Normanly & Abselson, 1989). We have previously shown that $tRNA^{fMet}$ containing the amber anticodon CUA accepts glutamine in vitro (Schulman & Pelka, 1985), and recent studies have indicated that the mischarged tRNA can initiate protein synthesis from an amber codon (Varshney & RajBhandary, 1990). In the present study we have directly demonstrated that $tRNA^{fMet}$ can initiate in vivo protein synthesis with two chemically different types of non-methionine amino acids. The methods described here provide a convenient and possibly general assay for further assessing the relationship between anticodon sequence and tRNA amino acid acceptor identity in vivo, as well as for examining the effects of sequence changes in other regions of the tRNA molecule on the specificity of aminoacylation in *E. coli*.

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Articles

Fluorescence Characterization of the Interaction of Various Transfer RNA Species with Elongation Factor Tu·GTP: Evidence for a New Functional Role for Elongation Factor Tu in Protein Biosynthesis†

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ABSTRACT: The ubiquity of elongation factor Tu (EF-Tu)-dependent conformational changes in aminoacyl-tRNA (aa-tRNA) and the origin of the binding energy associated with aa-tRNA·EF-Tu·GTP ternary complex formation have been examined spectroscopically. Fluorescein was attached covalently to the 4-thiouridine base at position 8 (s^4U-8) in each of four elongator tRNAs (Ala, Met-m, Phe, and Val). Although the probes were chemically identical, their emission intensities in the free aa-tRNAs differed by nearly 3-fold, indicating that the dyes were in different environments and hence that the aa-tRNAs had different tertiary structures near s^4U-8. Upon association with EF-Tu·GTP, the emission intensities increased by 244%, 57%, or 15% for three aa-tRNAs due to a change in tRNA conformation; the fourth aa-tRNA exhibited no fluorescence change upon binding to EF-Tu·GTP. Despite the great differences in the emission intensities of the free aa-tRNAs and in the magnitudes of their EF-Tu-dependent intensity increases, the emission intensity per aa-tRNA molecule was nearly the same (within 9% of the average) for the four aa-tRNAs when bound to EF-Tu·GTP. Thus, the binding of EF-Tu·GTP induced or selected a tRNA conformation near s^4U-8 that was very similar, and possibly the same, for each aa-tRNA species. It therefore appears that EF-Tu functions, at least in part, by minimizing the conformational diversity in aa-tRNAs prior to their beginning the recognition and binding process at the single decoding site on the ribosome. Since an EF-Tu-dependent fluorescence change was also observed with fluorescein-labeled $tRNA^{Phe}$, the protein-dependent structural change is effected by direct interactions between EF-Tu and the tRNA and does not require the aminoacyl group. The K_d of the $tRNA^{Phe}$·EF-Tu·GTP ternary complex was determined, at equilibrium, to be 2.6 μM by the ability of the unacylated tRNA to compete with fluorescent Phe-tRNA for binding to the protein. Comparison of this K_d with that of the Phe-tRNA ternary complex showed that in this case the aminoacyl moiety contributed 4.3 kcal/mol toward ternary complex formation at 6 °C but that the bulk of the binding energy in the ternary complex was derived from direct protein–tRNA interactions. The acetylation of Phe-$tRNA^{Phe}$ had a greater effect on its ternary complex K_d than did the formylation of Met-$tRNA_f^{Met}$, which suggests that N-blocked aa-tRNA species are prevented sterically from filling the site on EF-Tu that binds the α-amino group. The affinity of EF-Tu·GTP for both elongator and initiator Met-$tRNA^{Met}$ species has also been quantified.

Protein biosynthesis in prokaryotes is catalyzed by a nonribosomal protein termed elongation factor Tu (EF-Tu)[1] (Miller & Weissbach, 1977). EF-Tu binds tightly to aminoacyl-tRNA (aa-tRNA) in the presence of GTP to form an aa-tRNA·EF-Tu·GTP ternary complex (Miller & Weissbach, 1977; Johnson et al., 1986), and it is in this form that aa-tRNA begins the recognition and binding process at the ribosome. It is therefore reasonable to assume that EF-Tu somehow

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[1] Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; s^4U, 4-thiouridine; tRNA-Fl[8], adduct between 5-(iodoacetamido)fluorescein and the s^4U base of a tRNA at position 8; PEP, phosphoenolpyruvate.

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