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Initiation of protein synthesis from a termination codon

(gene regulation/initiation with glutamine/initiation with AUC/chloramphenicol acetyltransferase gene/amber mutants)

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ABSTRACT **We show that the amber termination codon UAG can initiate protein synthesis in *Escherichia coli*. We mutated the initiation codon AUG of the chloramphenicol acetyltransferase (CAT) gene to UAG (*CATam1*) and translated mRNA derived from the mutant CAT gene in *E. coli* S-30 extracts. A full-length CAT polypeptide was synthesized in the presence of $tRNA^{fMet}_{CUA}$, a mutant *E. coli* initiator tRNA which has a change in the anticodon sequence from CAU to CUA. Addition of purified *E. coli* glutaminyl-tRNA synthetase substantially stimulated synthesis of the CAT polypeptide. Thus, initiation of protein synthesis with UAG and $tRNA^{fMet}_{CUA}$ most likely occurs with glutamine and not methionine. The UAG codon also initiates protein synthesis *in vivo*. To eliminate a weak secondary site of initiation from AUC, the fifth codon, we further mutagenized the *CATam1* gene at codons 2 (GAG → GAC) and 5 (AUC → ACC). Transformation of *E. coli* with the resultant *CATam1.2.5* gene yielded transformants that synthesized CAT polypeptide and were resistant to chloramphenicol only when they were also transformed with the mutant $tRNA^{fMet}_{CUA}$ gene. Immunoblot analyses and assays for CAT enzyme activity in extracts from transformed cells indicate that initiation from UAG is efficient, 60–70% of that obtained from AUG. Initiation of protein synthesis from UAG using a mutant initiator tRNA allows tightly regulated expression of specific genes. This may be generally useful for overproduction in *E. coli* and other eubacteria of proteins which are toxic to these cells.**

Initiation of protein synthesis *in vivo* always uses the amino acid methionine or its derivative formylmethionine in eubacteria, mitochondria, and chloroplasts (1). The codon used for initiation, AUG, is also universal, although occasionally initiation by codons closely related to AUG and differing from it at one position such as UUG, GUG, CUG, AUA, ACG, AGG has been observed (1–4). However, the amino acid used for initiation is invariably methionine. The universal use of methionine as the initiating amino acid and of AUG as the initiating codon raises the question of whether protein synthesis *in vivo* can ever be initiated with an amino acid other than methionine and with a codon or codons that are not closely related to AUG.

By using *Escherichia coli* initiator tRNA misaminoacylated with valine or phenylalanine and then formylated, it has been shown *in vitro* that initiator tRNAs carrying these amino acids bind to the P (peptidyl-tRNA) site on *E. coli* ribosomes (5). Also, by using synthetic mRNAs such as poly(U) and poly(U,G) it has been shown that even noninitiator tRNAs such as Ac-Phe-tRNA and Ac-Trp-tRNA can initiate protein synthesis in *E. coli* extracts (6, 7). The relationship of these *in vitro* studies to the situation *in vivo* is, however, not known. There is no evidence in *E. coli* or any organism for initiation of protein synthesis with an amino acid other than methionine or with a codon that is not closely related to AUG. In eukaryotic systems it appears that protein synthesis may be initiated only with methionine. A yeast initiator tRNA aminoacylated with methionine is active in initiation *in vitro*, whereas the same tRNA aminoacylated with isoleucine is not (8).

During structure–function relationship studies of *E. coli* initiator tRNA (9–11) we generated a tRNA mutant in which the anticodon sequence CAU was changed to CUA. This mutant has the potential to read the amber termination codon UAG. Despite the fact that the mutant tRNA is aminoacylated with glutamine instead of methionine (11, 12), the aminoacylated mutant tRNA is formylated by *E. coli* methionyl-tRNA transformylase (B. L. Seong and U.L.R., unpublished observations) and it has features known to be important for directing it to the *E. coli* ribosomal P site (9). We have, therefore, used this mutant tRNA to ask whether protein synthesis can be initiated with UAG. Using chloramphenicolacetyl transferase (CAT) genes in which the initiator AUG codon has been changed to UAG, we show that UAG can initiate protein synthesis both *in vitro* and *in vivo* in *E. coli*. Initiation with UAG requires the presence of the mutant tRNA and appears surprisingly efficient *in vivo*.

MATERIALS AND METHODS

tRNA. The tRNA mutant used in this work and its isolation has been described previously (9–11).

Mutagenesis of CAT Genes. *CATam1* mutant was generated by Baik L. Seong (Massachusetts Institute of Technology), using the gapped duplex method of mutagenesis as described before (13). *CAT2.5* and *CATam1.2.5* were generated by phosphorothioate-based oligonucleotide mutagenesis (14) using a kit from Amersham. Efficiency of mutagenesis was greater than 90%. All mutants were characterized by sequence analysis.

Subcloning. Standard cloning techniques were used (15) and all clones/plasmid constructs were confirmed by sequence analysis. The vectors used for subcloning of the CAT, tRNA, and glutaminyl-tRNA synthetase (GlnRS) genes were based on pSP64 (16), pRSV (13), or pACYC (17). Plasmid pAC1 was generated by deletion of the T7 RNA polymerase gene from pACT7 obtained from S. Karnik (Massachusetts Institute of Technology).

***In Vitro* Translations.** S-30 extracts were prepared from *E. coli* MRE600 (18) and used as described previously (9). The incubation mixture (12.5 μl) for translation of CAT mRNAs (1 μg) synthesized with SP6 RNA polymerase (19) contained 10–15 μCi of [^{35}S]methionine (170–250 Ci/mmol; 1 Ci = 37 GBq), 4 units of RNasin (Promega), 0.4 mM glutamine, and 7.5 mM $MgCl_2$. The rest of the components were as described before (9, 10). Incubations were at 37°C for 1 hr. Aliquots (3.5 μl) were analyzed by electrophoresis on an SDS/15% polyacrylamide gel and the products were detected by autoradiography.

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Abbreviations: CAT, chloramphenicol acetyltransferase; GlnRS, glutaminyl-tRNA synthetase.

Immunoblot Analyses. Crude extracts were prepared from a 4-hr culture (1.2 ml) of *E. coli* transformants containing the various plasmids by resuspending the cell pellet in 200 μl of 1.5% SDS/40 mM Tris·HCl, pH 7.5/0.8 mM EDTA/8 mM 2-mercaptoethanol and heating the suspension at 90°C for 5 min. Protein concentrations were determined by using the Bradford dye binding assay (20). Total proteins (15 or 30 μg) were separated on SDS/15% polyacrylamide gels and electroblotted to polyvinylidine difluoride (PVDF) membranes (Millipore). CAT or β-lactamase was probed for with rabbit anti-CAT or anti-β-lactamase antibodies (obtained from 5 Prime → 3 Prime, Inc.) and detected with alkaline phosphatase-conjugated antibody to rabbit IgG (Promega) as the second antibody, using protocols provided by Millipore.

RESULTS

Mutations in the $tRNA^{fMet}$ and CAT Genes. Fig. 1 *Left* shows the *E. coli* initiator tRNA mutant used in these studies. The mutant tRNA has a change in the anticodon from CAU to CUA. It can be aminoacylated with glutamine instead of methionine, although it is a poorer substrate for *E. coli* GlnRS compared to $tRNA^{Gln}$ (11, 12). The mutant tRNA has the potential to read the amber termination codon UAG.

Fig. 1 *Right* shows the sites of mutation in the CAT gene. The mutants have changes in codon 1 from AUG to UAG (*CATam1*), in codons 2 and 5 from GAG to GAC and AUC to ACC, respectively (*CAT2.5*), and in codons 1, 2, and 5 (*CATam1.2.5*).

Translation of Wild-Type and Mutant CAT mRNAs in *E. coli* Extracts. Part A of Fig. 2 *Lower* (lanes 1–3) shows the results of *in vitro* translation in S-30 extracts directed by SP6 RNA polymerase transcripts derived from pSP64 *CAT* and pSP64 *CATam1*. An ≈24-kDa polypeptide, which corresponds to the size expected for CAT, is the predominant translation product of *CAT* mRNA (lane 2). As expected, this polypeptide is not present in translation products directed by *CATam1* mRNA; however, a slightly shorter polypeptide, ≈23.5 kDa, is present in much smaller amounts (lane 3). The 23.5-kDa polypeptide is also a translation product of *CAT* mRNA (lane 2) but is not seen clearly due to its closeness to the major 24-kDa polypeptide band. The 23.5-kDa polypeptide is related to CAT, since it is absent from a translation reaction to which no mRNA was added (lane 1) and it is immunoprecipitated by rabbit anti-CAT antibodies (data not shown). These results suggest that the 23.5-kDa polypeptide is derived by translation initiation from a weak secondary site which is located immediately downstream of, and in frame with, the major initiation codon AUG. The most likely site is the fifth codon, AUC, which differs from AUG in a single position (Fig. 2 *Upper*). Sequences upstream of this AUC codon conform to the Shine–Dalgarno consensus (1).

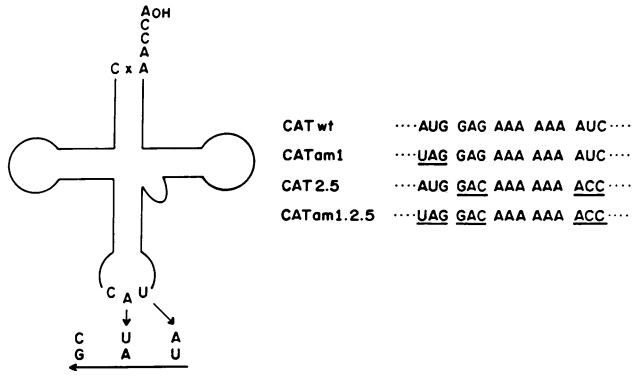


FIG. 1. (*Left*) Diagram of *E. coli* initiator tRNA, indicating the sites of mutation. (*Right*) Sequences near the initiation codon of various CAT mRNAs used in this work; wt, wild type. Underlined codons show the positions where the CAT gene was mutagenized.

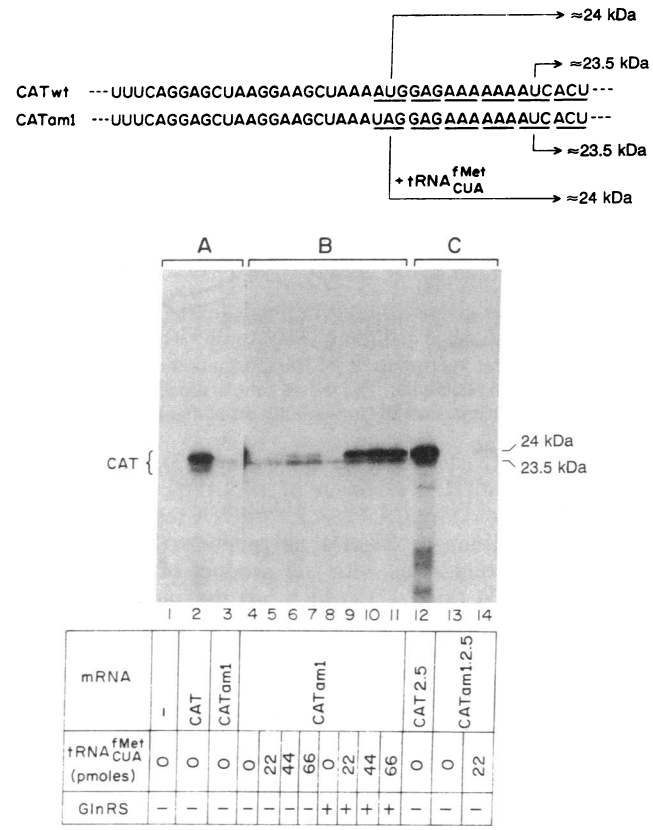


FIG. 2. (*Upper*) Sequences around the translational initiation sites of wild-type and *CATam1* mutant mRNAs. Arrows indicate the initiation sites and generation of ≈24-kDa and ≈23.5-kDa CAT polypeptides. (*Lower*) Autoradiographs of *in vitro* translation products fractionated on polyacrylamide gels and table of components in the reaction mixture. A, B, and C show results of separate *in vitro* translational reactions. Translations of wild-type and mutant CAT mRNAs in the absence (−) or presence (+) of ≈0.1 unit of GlnRS and of various amounts of $tRNA^{fMet}_{CUA}$ (0, 22, 44, or 66 pmol) were performed as described in *Materials and Methods*.

UAG Initiates Protein Synthesis in *E. coli* Extracts. Part B of Fig. 2 *Lower* shows that when the extracts are supplemented with $tRNA^{fMet}_{CUA}$, the amber codon in *CATam1* can initiate protein synthesis, as indicated by the synthesis of a 24-kDa polypeptide in addition to the 23.5-kDa polypeptide (compare lanes 5–7 to lane 4). Increasing the amount of $tRNA^{fMet}_{CUA}$ (22, 44, or 66 pmol) has only a marginal effect on synthesis of the 24-kDa polypeptide (compare lanes 5, 6, and 7). However, addition of exogenous GlnRS to reaction mixtures containing $tRNA^{fMet}_{CUA}$ leads to a significant stimulation in synthesis of the 24-kDa polypeptide (compare lanes 5, 6, and 7 to lanes 9, 10, and 11, respectively). GlnRS stimulation of synthesis of the 24-kDa polypeptide depends upon $tRNA^{fMet}_{CUA}$ (compare lane 8 to lanes 9, 10, and 11). Thus, initiation of protein synthesis *in vitro* from UAG in the *CATam1* mRNA requires $tRNA^{fMet}_{CUA}$ and uses glutamine instead of methionine.

To show that UAG can also initiate protein synthesis *in vivo* in *E. coli* (see below), we introduced further mutations in the *CATam1* gene at codons 2 (GAG → GAC) and 5 (AUC → ACC) to (*i*) eliminate the AUC initiation site, (*ii*) weaken the Shine–Dalgarno consensus sequence upstream of the AUC (Fig. 2 *Upper*), and (*iii*) rule out any possible initiation from GAG by $tRNA^{fMet}_{CUA}$. *In vitro* translation of the *CATam1.2.5* mRNA showed that introduction of mutations at codons 2 and 5 abolishes the weak translation initiation

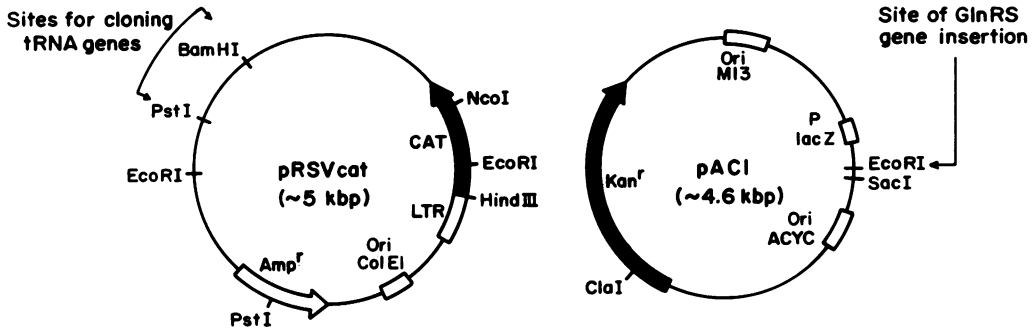


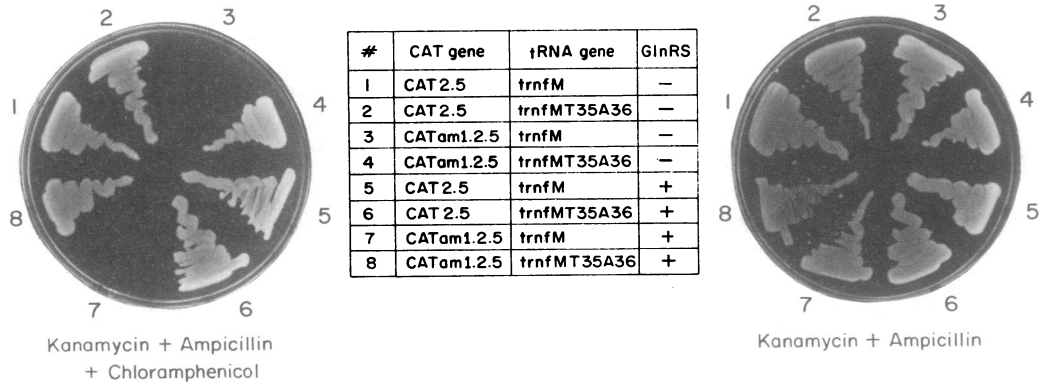
FIG. 3. Parent vectors used for transformation of *E. coli* CA274. kbp, Kilobase pairs; LTR, long terminal repeat; Ori, origin of replication; Amp^r, ampicillin resistance. The tRNA gene in the pRSV plasmid is in opposite orientation to the CAT gene. The GlnRS gene in the pAC1 plasmid is in opposite orientation to the kanamycin resistance (Kan^r) gene and to the *lac* promoter.

from codon 5 (part *C* of Fig. 2 *Lower*, lane 13). As in the case of *CATam1* mRNA, addition of $tRNA^{fMet}_{CUA}$ to translation reactions directed by *CATam1.2.5* mRNA results in synthesis of the full-length 24-kDa polypeptide (lane 14). This polypeptide comigrates with the product of translation of *CAT2.5* mRNA (lane 12), which has the same changes as *CATam1.2.5* at codons 2 and 5 but has AUG at codon 1 (Fig. 1 *Right*).

UAG Initiates Protein Synthesis *in Vivo* in *E. coli*. The stimulation of translation of *CATam1* mRNA by GlnRS (Fig. 2 *Lower*, compare lanes 5–7 with 9–11) suggested that aminoacylation of $tRNA^{fMet}_{CUA}$ by GlnRS may be limiting in its participation in initiation of protein synthesis. Therefore, to test whether UAG initiates protein synthesis *in vivo*, we used two plasmids, one consisting of the tRNA and CAT genes and the other the GlnRS gene (21). The tRNA genes and the CAT genes were cloned in a plasmid carrying the ColE1 replication origin and ampicillin resistance marker and the GlnRS gene was cloned in a low-copy-number plasmid carrying the ACYC replication origin and kanamycin-resistance marker (Fig. 3). The tRNA genes used were either the wild-type $tRNA^{fMet}$ gene *trnfM* (22) or the mutant *trnfMT35A36* (11). The CAT genes used were either *CAT2.5* or *CATam1.2.5*.

E. coli CA274 was transformed with either pAC1, a plasmid which carries a kanamycin resistance marker, or pACQS, which has the GlnRS gene inserted into *Eco*RI site of pAC1 (Fig. 3). Two sets of kanamycin-resistant transformants were isolated and each was transformed with four different pRSV plasmids carrying the $tRNA^{fMet}$ gene (wild type or the *T35A36* mutant) and the CAT gene (*CAT2.5* or *CATam1.2.5*). The eight different transformants were screened for growth on plates containing kanamycin and ampicillin as a control (Fig. 4 *Right*) or kanamycin, ampicillin, and chloramphenicol (Fig. 4 *Left*). All transformants grew on kanamycin and ampicillin; thus the transformants contain both plasmids. As expected, transformants carrying the *CAT2.5* gene and hence the wild-type allele in codon 1 are chloramphenicol resistant irrespective of whether the plasmid also carries the wild-type or the mutant *trnfM* gene (Fig. 4 *Left*, sectors 1, 2, 5, and 6). Transformants carrying the *CATam1.2.5* gene are also chloramphenicol resistant when they have the mutant *trnfMT35A36* gene (sectors 4 and 8) but not when they have the wild-type *trnfM* gene (sectors 3 and 7). Thus, in the presence of $tRNA^{fMet}_{CUA}$, UAG initiates protein synthesis in *E. coli*. Fig. 4 also shows that the chloramphenicol-resistance phenotype of transformants carrying the *CATam1.2.5* gene and the *trnfMT35A36* gene does not require overproduction of GlnRS in *E. coli* (compare sectors 8 and 4).

Initiation *in Vivo* with UAG Is Efficient. Immunoblot analysis (23) of total extracts (15 or 30 μg of protein) with rabbit anti-CAT antibodies was used to estimate the relative levels of CAT protein in the various transformants (Fig. 5). The following results are noteworthy. (*i*) The absence of any CAT protein in transformants containing the *CATam1.2.5* gene in combination with the *trnfM* gene (lanes 3 and 7) shows that initiation from UAG is absolutely dependent upon the presence of $tRNA^{fMet}_{CUA}$. (*ii*) Comparison of the relative signals obtained from transformants carrying the *CATam1.2.5* and *trnfMT35A36* genes (lanes 4 and 8) to those carrying the *CAT2.5* gene with either the *trnfM* gene (lanes 1 and 5) or the *trnfMT35A36* gene (lanes 2 and 6) shows that initiation *in vivo* with UAG is highly efficient, at least 50% of that obtained with AUG (compare lane 8, which shows signal from 30 μg



#	CAT gene	tRNA gene	GlnRS
1	CAT2.5	trnfM	–
2	CAT2.5	trnfMT35A36	–
3	CATam1.2.5	trnfM	–
4	CATam1.2.5	trnfMT35A36	–
5	CAT2.5	trnfM	+
6	CAT2.5	trnfMT35A36	+
7	CATam1.2.5	trnfM	+
8	CATam1.2.5	trnfMT35A36	+

FIG. 4. Screening of the various *E. coli* CA274 transformants for growth on 2YT agar plates (15) containing either kanamycin (25 μg/ml) plus ampicillin (50 μg/ml) plus chloramphenicol (30 μg/ml) (*Left*) or kanamycin (25 μg/ml) plus ampicillin (50 μg/ml) (*Right*). As indicated in the table (*Center*), the transformants streaked in the numbered sectors contained the following plasmids: 1 and 5, pRSV *CAT2.5 trnfM*; 2 and 6, pRSV *CAT2.5 trnfMT35A36*; 3 and 7, pRSV *CATam1.2.5 trnfM*; 4 and 8, pRSV *CATam1.2.5 trnfMT35A36*. In addition, 1–4 contained pAC1 and 5–8 contained pACQS with the GlnRS gene.

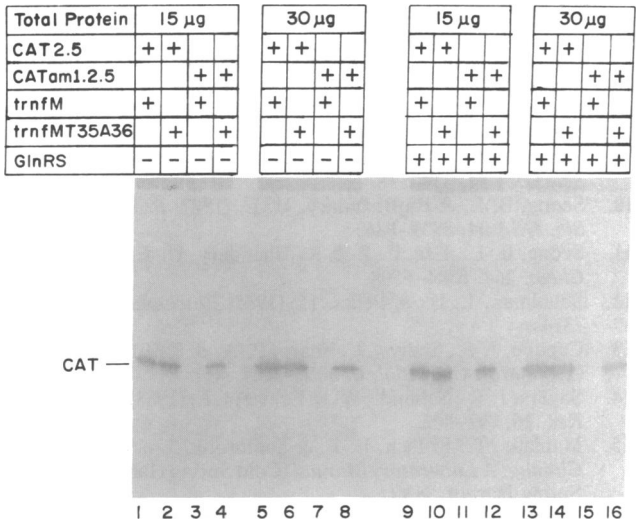


FIG. 5. Immunoblot analysis, using rabbit anti-CAT antibodies, of crude extracts (15 or 30 μg of protein) prepared from transformants 1–8 of Fig. 4 grown in the presence of kanamycin (25 μg/ml) and ampicillin (50 μg/ml).

of protein, with lane 2, which shows signal from 15 μg of protein). (*iii*) Overproduction of GlnRS results in a slight increase in CAT in all cases (compare lanes 1 and 5 with 9 and 13, 2 and 6 with 10 and 14, and 4 and 8 with 12 and 16, respectively) but not to a specific increase only in cells containing the *CATam1.2.5* and *trnfMT35A36* genes (also see below). The general increase in CAT in cells overproducing GlnRS is most likely due to increase in copy number of pRSV *CAT trnfM* plasmids. Immunoblot analysis of the same extracts also showed increased β-lactamase, another protein encoded by the pRSV *CAT trnfM* plasmid (Fig. 3), in cells overproducing GlnRS (data not shown).

A more quantitative measure of the efficiency of initiation with UAG was obtained by comparing CAT enzyme activity (13) in extracts made from the set of transformants containing the GlnRS plasmid. Results summarized in Table 1 show that efficiency of initiation with UAG is about 60–70% that with AUG. In addition, they show that synthesis of CAT polypeptide from the *CATam1.2.5* gene requires the presence of the *trnfMT35A36* gene.

DISCUSSION

We have shown that in the presence of $tRNA^{fMet}_{CUA}$, which is aminoacylated with glutamine *in vitro*, the amber termination codon UAG can initiate protein synthesis *in vivo* in *E. coli*. Thus, there is, *a priori*, no reason in *E. coli* for restricting the initiator codon to AUG and the initiating amino acid to methionine. Our results suggest that the main reason for AUG being used as the universal codon for initiation lies in its ability to base-pair with the anticodon of the initiator tRNA on the P site of the ribosome. In other words, once the ribosome has bound to a certain region on the mRNA, it is mostly the tRNA on the P site which selects the codon that is to be used for initiation and not the ribosome (24, 25). Work of R. Chattapadhyay, H. Pelka, and L. H. Schulman (personal communication) with other anticodon mutants of *E. coli* $tRNA^{fMet}$ supports this view.

Table 1. CAT specific activities in extracts of the various *E. coli* CA274 transformants

CAT gene	tRNA gene	CAT activity, units/μg protein
CAT2.5	*trnfM*	1.8
CAT2.5	*trnfMT35A36*	1.6
CATam1.2.5	*trnfM*	ND
CATam1.2.5	*trnfMT35A36*	1.1

One unit of activity is defined as 1 nmol of chloramphenicol converted to acetylchloramphenicol per min at 36°C. Extracts were prepared by lysozyme treatment of cells, containing the GlnRS plasmid, which were grown for 12 hr in the presence of ampicillin and kanamycin. The incubation mixture (100 μl) contained 160 μM [^{14}C]chloramphenicol, 800 μM acetyl coenzyme A, and 0.3 μg of protein from the extract. Incubation was for 10 min at 36°C. ND, not detectable.

Mutations in the anticodon sequence of elongator tRNAs often produce missense and nonsense suppressors which read codons different from those read by the corresponding wild-type tRNAs (26, 27). In spite of this apparent precedent, our finding that $tRNA^{fMet}_{CUA}$, an anticodon sequence mutant of $tRNA^{fMet}$, can initiate protein synthesis in *E. coli* with UAG is quite unexpected. The reason is that initiation of protein synthesis is a process that is quite distinct from the repetitive steps of elongation that follow it. The crucial steps in initiation all occur on the 30S ribosomal subunit, whereas the elongation reaction occurs on the 70S ribosome (24). Initiation requires the participation of several special protein factors and a special tRNA, the initiator tRNA, whose properties are different in many respects from those of elongator tRNAs (1, 28–30). It might, therefore, have been expected that the use of AUG as the codon and methionine as the amino acid for initiation was the result of a rigorous selection process during evolution which has preempted codons unrelated to AUG (see introduction) or amino acids other than methionine from participating in initiation. In fact, in a eukaryotic system, there are indications that protein synthesis may be initiated only with methionine (8).

The strong stimulation in translation of *CATam1* mRNA in cell-free extracts upon addition of *E. coli* GlnRS suggests that initiation *in vitro* with UAG involving $tRNA^{fMet}_{CUA}$ occurs with glutamine (Fig. 2, lanes 9–11). However, overproduction of GlnRS did not lead to a specific increase in CAT protein synthesis *in vivo* (Fig. 5). There are two possible explanations for this result: (*i*) The levels of GlnRS and the amounts of $tRNA^{fMet}_{CUA}$ produced from the multicopy pRSV plasmid (Fig. 3) in *E. coli* CA274 are such that aminoacylation of $tRNA^{fMet}_{CUA}$ with glutamine is not a limiting step for its participation in initiation of protein synthesis. (*ii*) Initiation *in vivo* involving $tRNA^{fMet}_{CUA}$ does not use glutamine. We favor the first possibility, since we showed previously that a mutant $tRNA^{fMet}$ which carries the same anticodon sequence change as $tRNA^{fMet}_{CUA}$, but has additional changes which make it a poorer substrate for GlnRS than $tRNA^{fMet}_{CUA}$, most likely inserts glutamine *in vivo* during suppression of amber codons (11).

If initiation *in vivo* with UAG uses glutamine, it may use glutaminyl-$tRNA^{fMet}_{CUA}$ or formylglutaminyl-$tRNA^{fMet}_{CUA}$. *In vitro*, the Gln-$tRNA^{fMet}_{CUA}$ can be formylated to fGln-$tRNA^{fMet}_{CUA}$ with Met-tRNA transformylase (B. L. Seong and U.L.R., unpublished observation), and other studies suggest that sites crucial for formylation of Met-$tRNA^{fMet}$ are located mostly in the acceptor stem (C. P. Lee, B. L. Seong, and U.L.R., unpublished observations). It is, therefore, likely that initiation of protein synthesis involving $tRNA^{fMet}_{CUA}$ occurs with formylglutamine. In any case, it would be interesting to see whether initiation with glutamine or formylglutamine instead of formylmethionine has any effect on the rates of turnover of the protein in *E. coli* (31).

Synthesis of CAT protein from the *CATam1.2.5* gene is absolutely dependent upon the presence of $tRNA^{fMet}_{CUA}$ (Figs. 4 and 5 and Table 1). Also, the level of CAT protein synthesized with UAG as the initiator codon is at least 50%, probably 60–70%, of that synthesized with AUG (Fig. 5 and Table 1). Together, these findings provide an approach (*i*) for tightly regulated expression of genes for proteins and (*ii*) for

overproduction in *E. coli* and other eubacteria of proteins which are toxic to these cells. By mutagenizing these genes such that translation of their mRNA is dependent upon the presence of $tRNA^{fMet}_{CUA}$, expression of the protein genes may be controlled at the transcriptional or the translational level or both. In the present work, the mutant $tRNA^{fMet}$ gene, *trnfMT35A36*, is constitutively expressed; however, there are well-established systems for regulating the expression of tRNA genes in *E. coli* (32). In this regard, it is also worth noting that the mutant $tRNA^{fMet}_{CUA}$ works exclusively in initiation (11) and can, therefore, be expressed at high levels without any problems due to suppression of normal UAG termination codons.

Finally, the present work represents an important step for studies on structure–function relationships of *E. coli* initiator tRNA. It provides an assay for *in vivo* function of mutant initiator tRNAs. The large number of mutants of *E. coli* $tRNA^{fMet}$ that we have generated can now each be combined with the *T35A36* mutation and their ability to support synthesis of the CAT protein *in vivo* from the *CATam1.2.5* gene can be analyzed.

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