A 2-Thiouridine Derivative in $tRNA^{Glu}$ Is a Positive Determinant for Aminoacylation by *Escherichia coli* Glutamyl-tRNA Synthetase†

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ABSTRACT: Early investigations into the interaction between *Escherichia coli* glutamyl-tRNA synthetase (GluRS) and $tRNA^{Glu}$ have implicated the modified nucleoside 5-[(methylamino)methyl]-2-thiouridine in the first position of the anticodon as an important contact for efficient aminoacylation. However, the experimental methods employed were not sufficient to determine whether the interaction was dependent on the presence of the modification or simply involved other anticodon loop–nucleotides, now occluded from interaction with the synthetase. Unmodified *E. coli* $tRNA^{Glu}$, derived by in vitro transcription of the corresponding gene, is a poor substrate for GluRS, exhibiting a 100-fold reduction in its specificity constant (k_{cat}/K_M) compared to that of $tRNA^{Glu}$ prepared from an overproducing strain. Through the use of recombinant RNA technology, we created several hybrid tRNAs which combined sequences from the in vitro transcript with that of the native tRNA, resulting in tRNA molecules differing in modified base content. By in vitro aminoacylation of these hybrid tRNA molecules and of tRNAs with base substitutions at positions of nucleotide modification, we show conclusively that the modified uridine at position 34 in $tRNA^{Glu}$ is required for efficient aminoacylation by *E. coli* GluRS. This is only the second example of a tRNA modification acting as a positive determinant for interaction with its cognate aminoacyl-tRNA synthetase.

A common feature of transfer RNA is the presence of posttranscriptional modifications including a few modified nucleotides which are almost universally conserved in type and location. Although the question of what role these modifications play in vivo has been under investigation for many years, it is not possible to define for a single tRNA what specific contributions are made. In general terms, modifications located outside the anticodon are thought to provide structural stability and/or to facilitate interaction with enzymes other than aminoacyl-tRNA synthetases. Modifications located within the anticodon loop are believed to increase translational efficiency and accuracy by strengthening the codon–anticodon pairing while weakening wobble interactions [reviewed by Björk (1992)].

Early work to examine the involvement of modified nucleotides in tRNA recognition by aminoacyl-tRNA synthetases relied on the isolation of mutant bacterial strains deficient in the synthesis of specific tRNA modifications. That such strains were viable was indirect proof that at least some of the modified nucleotides are nonessential to tRNA function. Antisuppressor mutations, which decreased readthrough of stop codons by suppressor tRNAs, were found to cause undermodification of the wobble base in the tRNA anticodon in *Escherichia coli* (Sullivan & Bock, 1985; Sullivan et al., 1985) and *Schizosaccharomyces pombe* (Grossenbacher et al., 1986; Heyer et al., 1984). Interpretation of these results in terms of tRNA identity (the set of positive and negative elements in the tRNA structure which define a single amino acid acceptor type) is complicated by the fact that suppressor function in vivo involves translational efficiency as well as tRNA aminoacylation.

The ability to make tRNA devoid of modified nucleotides by in vitro transcription allowed the examination of the properties of unmodified tRNA in aminoacylation in vitro (Sampson & Uhlenbeck, 1988). The initial experiments led to the general expectation that all tRNAs devoid of modified nucleotides could be aminoacylated as only small differences were seen in the kinetic parameters of in vivo isolated tRNA and in vitro tRNA transcripts. However, studies with *E. coli* $tRNA_2^{Ile}$ showed that lysidine, a modified cytidine in the wobble position of the anticodon, acts as a positive determinant for aminoacylation by the cognate synthetase (Muramatsu et al., 1988). Extensive experiments with the transcript of yeast $tRNA^{Asp}$ revealed that the unmodified tRNA is more efficiently misacylated by arginyl-tRNA synthetase than is the native tRNA, leading to the conclusion that the modified nucleotides present in vivo provide negative determinants for misacylation by noncognate synthetases (Perret et al., 1990). As this is the only system rigorously tested to date for increased misacylation of tRNA transcripts in vitro, it is not possible to state whether this is a general role of modified nucleotides in tRNA. More recent experiments indicate that, compared to native *E. coli* $tRNA^{Lys}$, the corresponding in vitro transcript is aminoacylated at a reduced rate by lysyl-tRNA synthetase (Tamura et al., 1992); however, this property has not yet been attributed to any specific modified nucleotide.

What is known about the identity of *E. coli* $tRNA^{Glu}$? No systematic studies with mutant tRNAs with the aim of answering this question are available to date. GluRS and glutaminyl-tRNA synthetase share a close evolutionary relationship with high sequence homology within regions of glutaminyl-tRNA synthetase known to be in contact with the acceptor stem of $tRNA^{Gln}$ (Breton et al., 1990; Rould et al., 1989; Perona et al., 1989). The presumed structural homology suggests that the two enzymes interact with cognate tRNA

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in a similar fashion. Thus, it is a reasonable prediction that identity elements in $tRNA^{Glu}$ for GluRS recognition also reside in the acceptor stem and anticodon regions. Mutational changes in the first and third base of the anticodon or in the discriminator (position 73) did not cause loss of glutamylation in vivo, but the anticodon mutations correlated with increased misacylation with glutamine (Normanly et al., 1990; Sherman et al., 1992). The importance of the anticodon for glutamylation has been suggested in early chemical modification studies which implicated 5-[(methylamino)methyl]-2-thiouridine (mnm^5s^2U),[1] the first anticodon base, in the interaction with GluRS in vitro. Experiments with both unfractionated tRNA as well as purified $tRNA^{Glu}$ showed that treatment of tRNA with cyanogen bromide caused a decrease in glutamate acceptance (Saneyoshi & Nishimura, 1971; Agris et al., 1973; Seno et al., 1974) and an alteration of the kinetic parameters of aminoacylation (Kern & Lapointe, 1979). Interaction at this position in $tRNA^{Glu}$ with GluRS was also suggested by circular dichroism spectra of the GluRS–$tRNA^{Glu}$ complex (Willick & Kay, 1976) and by a reduction of glutamate acceptance in tRNA after exposure to near-ultraviolet light (Carré et al., 1974; Thomas et al., 1981).

There are at least two enzymes in *E. coli* that utilize $tRNA^{Glu}$ uniquely as a substrate, GluRS (Lapointe & Söll, 1972) and glutamyl-tRNA reductase which converts the activated glutamate of Glu-$tRNA^{Glu}$ to glutamate-1-semialdehyde (Verkamp et al., 1992). Our interest in determining how these enzymes interact with $tRNA^{Glu}$ led us to the studies described here. The finding that the wild-type in vitro transcript of $tRNA^{Glu}$ was poor substrate for glutamylation prompted an investigation into the probable role of modified nucleotides of $tRNA^{Glu}$ in recognition by GluRS by utilizing two approaches. The first method involved mutagenesis of a synthetic $tRNA^{Glu}$ gene at positions where posttranscriptional modification occurs. The $tRNA^{Glu}$ mutants with conservative base substitutions at positions normally modified were overproduced in vivo and purified for in vitro aminoacylation by GluRS to allow quantitation of the kinetic effect of each individual position of modification. In a complementary approach, hybrid tRNA variants were constructed in vitro by combination of in vitro transcript (unmodified) sequences with native (modified) sequences using a recombinant RNA technique involving site-directed cleavage of the tRNAs with ribonuclease H, followed by reconstruction with T4 RNA ligase (Hayase et al., 1992). The initial velocities of aminoacylation by GluRS were determined for these hybrid tRNA constructs to directly correlate undermodification with reduced recognition by GluRS.

EXPERIMENTAL PROCEDURES

Materials. L-[U-^{14}C]Glutamic acid (266 mCi/mmol), [γ-^{32}P]ATP (6000 Ci/mmol), and [5′-^{32}P]pCp (3000 Ci/mmol) were products of Amersham. *E. coli* RNase H was from Takara Biochemicals. T7 RNA polymerase was a generous gift of D. Jeruzalmi. *E. coli* $tRNA^{Glu}$ for hybrid construction and aminoacylation controls was from Oak Ridge National Laboratory. GluRS was purified from an overproducing *E. coli* strain (Lapointe et al., 1985) using a modified purification scheme to a specific activity of 2000 units·mg^{-1} (K. C. Rogers and D. Söll, manuscript in preparation). The chimeric oligonucleotides were synthesized according to Hayase et al. (1992). The in vitro transcript of *E. coli* $tRNA^{Glu}$ was synthesized and purified as described (K. C. Rogers and D. Söll, manuscript in preparation).

Overexpression of Wild-Type and Mutant tRNAs. All mutants were made by site-directed mutagenesis (Kunkel, 1985) of a wild-type $tRNA^{Glu}$ gene constructed for in vitro transcription (K. C. Rogers and D. Söll, manuscript in preparation). The entire $tRNA^{Glu}$ transcription cassette was recloned from pUC2119 into pGFIB (Normanly et al., 1986) as an *Eco*RI/*Pst*I fragment for constitutive expression of wild-type or mutant tRNA from the *lpp* promoter. *E. coli* strain DH5α was used for expression of the tRNAs. Unfractionated tRNA was purified (Perona et al., 1988) from 1-L cultures grown in LB medium containing 0.1 mg/mL ampicillin. Cultures were incubated for at least 4 h at saturation to allow for tRNA modification enzymes to modify fully the overproduced tRNA. Overproduction was estimated visually on polyacrylamide gels as well as by aminoacylation of crude tRNA. After all large-scale growths, plasmid DNA was isolated and sequenced to confirm the presence of the mutation(s). The overproduced tRNAs were purified by electrophoresis on 20% nondenaturing polyacrylamide gels (Kuchino et al., 1990) and could be aminoacylated by GluRS to >1200 pmol/A_{260} unit.

Kinetic Analysis of Transcript, Native, and Mutant tRNAs. Aminoacylation assays to determine the kinetic parameters of $tRNA^{Glu}$ variants were performed as described (Lapointe et al., 1985). The reactions contained tRNA and GluRS (at the concentrations indicated in Table I) and equal amounts of unlabeled Glu and [^{14}C]Glu for a total assay concentration of 0.4 mM glutamate. Five tRNA concentrations were used per mutant substrate with five time points taken in triplicate. The data were analyzed by both Eadie–Hofstee and Lineweaver–Burk plots.

Construction of Hybrid tRNA Variants. To direct single-site cleavages by RNase H (Hayase et al., 1990) at defined sites in native and in vitro transcript $tRNA^{Glu}$, chimeric oligonucleotides (consisting of 2′-deoxy- and 2′-*O*-methyl nucleotides) were used as described by Hayase et al. (1992). The oligonucleotide sequences are as follows: (1) 5′-CmCmGmCmCmGmTmGmAmAmAmGmGGCGGmTmGmTmCm, (2) 5′-GmCmCmGmUmGmAAAGGmGmCmGmGmUmGmUmCmCm, and (3) 5′-GmCmCmGTGAAmAmGmGmGmCmGmGmUmGmUmCmCm. The sites of cleavage affected by each of these oligonucleotides are illustrated in Figure 1. The resulting tRNA half-molecules were resolved by electrophoresis on 8 M urea–15% polyacrylamide gels, visualized by UV shadowing, excised, eluted, and precipitated with ethanol. Ligation of tRNA half-molecules was as described (Hayase et al., 1992), except that a 1:1 molar ratio of 5′:3′ halves was used in the annealing mixture. The products were resolved and purified by electrophoresis on denaturing 15% polyacrylamide gels as described above and sequenced (Donis-Keller, 1979).

Initial Aminoacylation Velocity Determination for the Hybrid tRNAs. Aminoacylation assays were performed as described (Lapointe et al., 1985) with 1.33 μM tRNA, 22.2 nM GluRS, and 31.3 μM [^{14}C]Glu. Initial aminoacylation velocities were calculated from four to five time points (taken in triplicate) in which the data remained linear (15, 30, 45, and 60 s for good substrates; 0.5, 1, 2, 3, and 4 min for poor substrates).

Magnesium-Catalyzed Cleavage of $tRNA^{Glu}$. 5′- or 3′-^{32}P-labeled in vitro transcribed or native $tRNA^{Glu}$ (1.0 μM) in 10 mM Tris-HCl (pH 7.5) was incubated for 4 h at 37 °C with or without 20 mM $MgCl_2$. The products were resolved

[1] Abbreviations: D, dihydrouridine; T, ribothymidine; m^2A, 2-methyladenosine; mnm^5s^2U, 5-[(methylamino)methyl]-2-thiouridine; ψ, pseudouridine; Nm, 2′-*O*-methyl nucleoside.

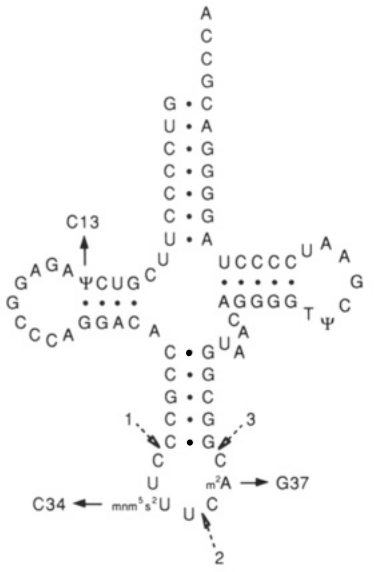

FIGURE 1: Cloverleaf representation of $tRNA^{Glu}$ highlighting base modifications, RNase H cleavage sites, and mutations. The sites of oligonucleotide-addressed RNase H cleavage are indicated by arrows labeled with the numbers used to represent the chimeric oligonucleotides under Experimental Procedures. Sites of mutation are indicated by arrows from the wild-type base to the resulting mutation.

Table I: Kinetic Parameters for in Vitro Transcript and Overexpressed Wild-Type and Mutant tRNAs

substrate	[tRNA] (μM)[a]	[GluRS] (μM)	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M
$tRNA^{Glu}$	0.08–0.8	0.5	0.38	3.6	1.0
$tRNA^{Glu}$ transcript	0.5–5.0	5.0	20	1.9	0.01
U13 → C $tRNA^{Glu}$	0.3–3.0	0.6	2.8	7.4	0.28
U34 → C $tRNA^{Glu}$	0.5–15.0	5.0	22	1.7	0.01
A37 → G $tRNA^{Glu}$	0.3–3.0	3.0	5.0	2.2	0.04
$tRNA^{Glu}$ (published)[b]	0.05–1.0	0.5	0.1	1.9	2.0

[a] Range of tRNA concentration used to determine kinetic parameters. [b] The values presented here are from Kern et al. (1979) for a commercial $tRNA^{Glu}$ preparation.

by electrophoresis on an 8 M urea–15% polyacrylamide gel alongside the partial RNase T_1-digested or alkaline hydrolyzed 5′-^{32}P-labeled $tRNA^{Glu}$ transcript or native RNA and visualized by autoradiography (Donis-Keller, 1979).

RESULTS

Aminoacylation Characteristics of $tRNA^{Glu}$ "Modification Mutants". There are five modified bases in *E. coli* $tRNA^{Glu}$: the universally conserved T54 and Ψ55 in the T loop, Ψ13 in the D stem, and mnm^5s^2U34 and m^2A37 in the anticodon loop (Figure 1). The kinetic parameters measured for the native tRNA and the unmodified $tRNA^{Glu}$ transcript are shown in Table I. A comparison of these data shows a 100-fold decrease in the specificity constant (k_{cat}/K_M) for the $tRNA^{Glu}$ transcript relative the overproduced tRNA. Our wild-type $tRNA^{Glu}$ purified from an overproducing strain exhibited a 2-fold decrease in k_{cat}/K_M compared to commercial preparations (Lapointe & Söll, 1972; Willick & Kay, 1976; Kern et al. 1979). This is likely to be due to heterogeneity in the degree of modification of the overproduced tRNA, since tRNA-modification enzymes may not be able to keep up with increased tRNA synthesis (Gefter & Russel, 1969) as recently documented for overproduced $tRNA_2^{Gln}$ (Perona et al., 1988).

In order to identify the modification positions responsible for the dramatic decrease in k_{cat}/K_M for the $tRNA^{Glu}$ transcript, mutant tRNAs with conservative substitutions at

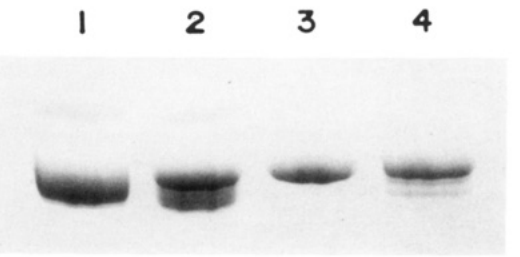

FIGURE 2: Analysis of overproduction and purification of $tRNA^{Glu}$ samples. Samples as indicated below were resolved on 7 M urea–12% polyacrylamide gel and visualized with toluidine blue staining. Lanes: 1, unfractionated RNA from strain DH5α; 2, unfractionated RNA from DH5α transformed with the $tRNA^{Glu}$ expression construct; 3, $tRNA^{Glu}$ purified by nondenaturing gel electrophoresis from the overproducing strain; 4, purified $tRNA^{Glu}$ from Oak Ridge National Laboratory.

positions of base modification were made and overproduced in vivo. Each of the bases, which are targets for modification in vivo, was mutated to the alternate purine or pyrimidine. As the same posttranscriptional modifications of the wild-type tRNA cannot be made on the substituted base in the mutant tRNA, it is assumed that the mutant tRNAs are unmodified at the substituted positions. For three of the mutants the tRNAs could be overexpressed to 40–50% of total cellular RNA (Figure 2). Thus, an accurate determination of K_M and k_{cat} could be made for the mutant tRNAs without significant contamination by wild-type $tRNA^{Glu}$ present in natural abundance. However, the $tRNA^{Glu}$ mutated in the universally conserved base Ψ55 could not be overproduced in the same manner. Therefore, three $tRNA^{Glu}$ mutants shown in Figure 1 were studied further.

The kinetic data for aminoacylation of the mutant tRNAs by GluRS are shown in Table I. While all of the tRNA mutants were affected in K_M and k_{cat} for glutamylation, it is clear that only the $mnm^5s^2U \rightarrow C34$ change reduced the overall specificity constant of the mutant tRNA by the same magnitude as that measured for the transcript. The similarity in the overall specificity constant of the transcript and $mnm^5s^2U \rightarrow C34$ mutant is unlikely to be a coincidental result of two different impediments to aminoacylation by GluRS as the individual kinetic parameters of K_M and k_{cat} are within experimental error for the two substrates, indicating similar effects on binding and catalysis. These data suggest that the modified nucleoside which is required for efficient aminoacylation of $tRNA^{Glu}$ by GluRS is the anticodon modification mnm^5s^2U34.

Initial Aminoacylation Velocities of Modification-Deficient Hybrid $tRNA^{Glu}$ Variants. Although the kinetic analysis of the tRNA mutants discussed above suggests that the modification found in the first anticodon position of $tRNA^{Glu}$ is directly involved in aminoacylation by GluRS, the possibility exists that the C34 mutation presents a strong negative determinant for GluRS recognition that mimics the kinetic effect of the unmodified $tRNA^{Glu}$ transcript. To address this possibility, we created hybrid tRNAs of unmodified sequence (in vitro transcripts) and modified sequence (native $tRNA^{Glu}$) by a recombinant RNA technique utilizing site-directed cleavage of the tRNAs with RNase H, followed by reconstruction with T4 RNA ligase (Hayase et al., 1992) (Figure 3). Using this technique, it was possible to isolate tRNA in which modified nucleotides are directly replaced with the corresponding unmodified nucleotide. Both the transcript $tRNA^{Glu}$ and native $tRNA^{Glu}$ were cleaved after either nucleotide 31, 35, or 38 (Figure 1) to generate tRNA fragments. The isolated tRNA fragments were then annealed

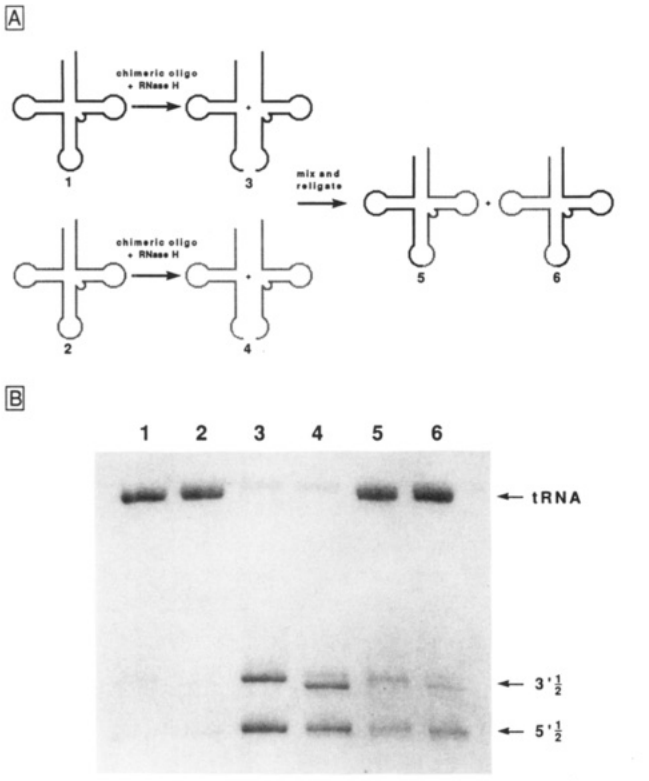

FIGURE 3: Scheme for constructing hybrid tRNAs. (A) General scheme illustrating arbitrary cleavage of native (dark lines) and in vitro transcript tRNAs (light lines) in the anticodon loop, followed by mixed ligations of transcript and native tRNA half-molecules. (B) From a hybrid tRNA construction using chimeric oligonucleotide 2, products 1–6 [as indicated in (A)] were resolved on a denaturing 15% polyacrylamide gel, and the RNA was stained with toluidine blue.

and ligated to form full-length tRNAs which were purified away from any remaining fragments by gel electrophoresis. For example, to produce a tRNA in which the Ψ13 was replaced by U13, the 5′ half of the transcript, produced by site-specific cleavage after residue 31, was ligated to the corresponding 3′ half produced from the native tRNA. The resulting tRNA is referred to as [U13]tRNA^{Glu}, with the alternate tRNA variant produced from this cleavage and ligation referred to as [U34,A37,U54,U55]tRNA^{Glu}. For each cleavage site, control tRNAs were produced in which native 5′- and 3′-fragments were religated. These tRNAs are referred to as [31/32]tRNA^{Glu}, [35/36]tRNA^{Glu}, and [38/39]-tRNA^{Glu}.

The initial aminoacylation velocity for each of the tRNA variants was measured under conditions which gave a linear portion of the aminoacylation curve for all substrates. The in vitro transcript of tRNA^{Glu} shows a 10-fold reduction in initial velocity of aminoacylation when compared to the fully modified native tRNA (Table II). The tRNA variants that lacked modifications in either the D stem (Ψ13) or T loop (T54 and Ψ55) had initial velocities identical to that of the native tRNA. The tRNA construct which was unmodified at positions 37, 54, and 55 exhibited a slightly reduced initial aminoacylation velocity versus the native (14.2 versus 16.7 min^{-1}). This is presumably not significant as the control tRNAs, which were reconstructed from native tRNA fragments, also had slight reductions in rate (14.6–15.2 min^{-1}). However, the tRNA construct in which the mnm^{5}s^{2}U modification is replaced by the unmodified U34 in addition to the Ψ → U13 substitution displayed a 10-fold decrease in initial aminoacylation velocity to match that measured for

Table II: Aminoacylation Capacities and Initial Aminoacylation Velocities for Hybrid tRNA^{Glu} Variants

tRNA^{Glu} variant	Glu acceptance (pmol/A_{260})	initial velocity (min^{-1})
native	1248	16.7
transcript	862	1.2
U13	1073	16.9
U13, U34	1012	1.7
U54, U55	1096	16.6
U13, U34, A37	912	1.3
U34, A37, U54, U55	990	1.5
A37, U54, U55	1014	14.2
31/32	1027	14.6
35/36	1138	15.2
38/39	1088	14.9

the fully unmodified in vitro transcript. The possibility that this is a synergistic effect due to the loss of modification at both position 13 and position 34 was excluded as the tRNA construct deficient in modification at position 34, in addition to 37, 54, and 55, resulted in an equally impaired substrate. Since the initial aminoacylation velocities of these substrates matched that of the in vitro transcript, the full defect of the transcript can be attributed to the loss of the 5-[(methylamino)-methyl]-2-thio group from mnm^{5}s^{2}U34. Therefore, the kinetic effect measured for the in vitro transcribed tRNA^{Glu} (Table I) is directly related to the loss of this modification.

To address the question of whether the modification at position 34 is directly recognized by GluRS, or simply that modification at this position is important to maintain an anticodon loop conformation which permits proper presentation to GluRS of other recognition elements in the tRNA, we probed the structure of native tRNA^{Glu} and the unmodified transcript in a Mg^{2+}-catalyzed cleavage assay. In the course of our study, we discovered that the phosphodiester bond between U35 and C36 is highly susceptible to cleavage in the presence of Mg^{2+} ions. Analysis by gel electrophoresis revealed no differences in the specificity or yield of cleavage of both native and in vitro transcript tRNA^{Glu} (data not shown). Since the coordination of metal ions in RNA molecules is highly dependent on tertiary structure [e.g., Reid and Cowan (1990)], and Pb^{2+}-catalyzed cleavage has been used to assess tRNA folding [e.g., Behlin et al. (1990)], this result indicates that the lack of modification in position 34 does not significantly perturb the structure of the anticodon loop. We therefore conclude that GluRS directly recognizes the modification on mnm^{5}s^{2}U34 and not some conformation of the tRNA uniquely brought about by the modification.

DISCUSSION

These results demonstrate a requirement for the modified nucleoside 5-[(methylamino)methyl]-2-thiouridine in the anticodon (position 34) of tRNA^{Glu} for efficient aminoacylation in vitro by *E. coli* GluRS. The unmodified tRNA^{Glu} transcript has a 50-fold increased K_M and a 100-fold decrease in specificity constant in comparison to the in vivo overproduced tRNA. However, the presence of this modification is not essential in vivo, as the amber suppressor derived from tRNA^{Glu} (with a C in position 34) has at least partial glutamate identity (Normanly et al., 1990; Sherman et al., 1992). Additional support for the observation that the fully modified mnm^{5}s^2-U34 is not essential for glutamylation in vivo is the viability of tRNA modification-deficient strains which contain tRNA^{Glu} only partially modified at this position. However, these modification-deficient strains show reduced growth rates which could be a direct result of less efficient aminoacylation as well

as reduced translational efficiency of the undermodified tRNA (Sullivan et al., 1985; Elseviers et al., 1984; Marinus et al., 1975; Hagervall et al., 1987). Although GluRS recognition clearly involves other identity elements outside of this anticodon modification, the dramatic decrease in the relative specificity constant for aminoacylation of the $tRNA^{Glu}$ transcript suggests that the U34 modification plays a major role in discrimination in vivo.

The in vivo made $tRNA^{Glu}$ mutants used for kinetic studies all show an increased K_M for glutamylation, although not as dramatic as that seen for the C34 mutant. The G37 mutant has an overall specificity constant decreased by 25-fold, suggesting that GluRS prefers the wild-type sequence at this position. The fact that the $\Psi \rightarrow$ C55 mutant could not be overexpressed in vivo suggests that this position of a conserved modification in *E. coli* tRNAs may be required for proper processing or folding. Perhaps more surprising is the effect of the $\Psi \rightarrow$ C13 mutation on the aminoacylation rate; although the K_M for this mutant is increased, the k_{cat} is greater than that of the wild-type $tRNA^{Glu}$. Replacement of Ψ by U at stem–loop junctions is thought to increase the flexibility of tRNA (Davis & Poulter, 1991); the observed elevation in k_{cat} may be related to higher conformational mobility, allowing easier aminoacylation by GluRS. However, this suggested increased flexibility could be detrimental in vivo, as an amber suppressor $tRNA^{Glu}$ containing the additional $\Psi \rightarrow$ C13 mutation shows a substantial decrease in suppressor efficiency compared to the parental suppressor (K. Rogers, unpublished results).

The importance of the contribution of the modified U34 in GluRS recognition in vivo is also supported by the observation that *E. coli* does not have the other potential isoacceptor for glutamate (anticodon CUC), relying instead on wobble reading of GAG glutamate codons (Komine et al., 1990). This potential isoacceptor, when generated by mutagenesis, is an equally poor substrate for aminoacylation in vitro as the unmodified tRNA transcript (Table I). Translation of GAG codons in vivo is dependent on wobble pairing by $tRNA^{Glu}$ (anticodon mnm^5s^2UUC), but the 2-thiol group is known to decrease the ability of U34 to form wobble pairs with G (Sekiya et al., 1969; Agris et al., 1973). This is consistent with the observation that the use of GAG codons in *E. coli* genes correlates with proteins expressed in low abundance (Sharp & Li, 1986). The requirement for U34 of $tRNA^{Glu}$ to be modified for efficient aminoacylation and the preference for unmodified tRNA in GAG codon translation reveals a dual role for nucleotide modification in protein synthesis as two mechanisms of translational control stem from one anticodon position. This provides the rationale for the observation of both modified and unmodified forms of $tRNA^{Glu}$ in vivo (Tremblay & Lapointe, 1986) and may explain the inability to isolate a strain completely deficient in mnm^5s^2U34 synthesis (Björk et al., 1987).

The glutamate system represents the second tRNA acceptor type for which a specific modified nucleotide acts as a positive recognition element for the cognate aminoacyl-tRNA synthetase. In the isoleucine system, where lysidine-34 is important, the unmodified anticodon allows the $tRNA_2^{Ile}$ to be misacylated in vitro by MetRS (Muramatsu et al., 1988). The recognition of mnm^5s^2U34 by GluRS may have evolved in a similar way to prevent both $tRNA^{Gln}$ (anticodons s^2UUG and CUG) misacylation by GluRS and $tRNA^{Glu}$ misacylation by glutaminyl-tRNA synthetase. The method of discrimination between $tRNA^{Glu}$ and $tRNA^{Gln}$ is of particular interest as the absence of glutaminyl-tRNA synthetase in Gram-positive eubacteria and organellar systems necessitates misacylation of $tRNA^{Gln}$ species by GluRS, followed by enzymatic amidation of glutamate to glutamine (Schön et al., 1988); however, such misacylation would be intolerable in a Gram-negative organism. In Gram-positive and organellar systems, the only $tRNA^{Gln}$ isoacceptor is anticodon UUG, while in Gram-negative systems both UUG and CUG isoacceptors for glutamine are found. It is plausible, therefore, that *E. coli* GluRS has evolved a very efficient means of discrimination against overlap in anticodon recognition, with C34 being a strong negative determinant and the hypermodified U34 an important positive determinant for glutamylation. This is supported by the finding that the amber suppressor derived from $tRNA^{Glu}$ (anticodon CUA) inserts 30–50% glutamine in vivo as a result of reduced competition for the tRNA by GluRS (Normanly et al., 1990; Sherman et al., 1992) and is an extremely poor substrate for aminoacylation in vitro by GluRS (K. Rogers, unpublished results).

The anticodon modification nmn^5s^2U34 is not unique to $tRNA^{Glu}$ in *E. coli*; it is also present in $tRNA^{Lys}$ and may be responsible for the decreased rate of aminoacylation seen with the in vitro transcript of $tRNA^{Lys}$ in comparison to native $tRNA^{Lys}$ (Tamura et al., 1992). If either GluRS or LysRS discriminated solely in favor of tRNAs with this modification in the anticodon, this would result in misacylation. However, although many synthetic amber suppressors are mischarged with lysine in vivo (Normanly et al., 1990; Sherman et al., 1992), the amber suppressor $tRNA^{Glu}$ inserts glutamate and glutamine but not lysine; even with bias toward lysine identity added by a G73 $\rightarrow$ A change in the discriminator nucleotide (McClain et al., 1990; Tamura et al., 1992), glutamate identity is retained (Sherman et al., 1992). This strongly suggests that $tRNA^{Glu}$ contains negative determinants for lysine identity outside of the anticodon which prevent misacylation by LysRS. Through mutation of amber suppressor tRNAs it has been shown that the discriminator base A73, the C3–G70 pair, and U35 are important for $tRNA^{Lys}$ identity (McClain & Foss, 1988; McClain et al., 1990). In addition, G45 may contribute to tRNA recognition by LysRS since this position on a $tRNA^{Phe}$ background confers lysine identity (McClain et al., 1988). As all of these positions except U35 differ in $tRNA^{Glu}$, there are several potential negative determinants for lysine identity which may present misacylation of $tRNA^{Glu}$ by LysRS.

The synthesis of the modified nucleoside mnm^5s^2U34 is complex, involving at least four enzymatic activities (Hagervall et al., 1987). As unique elements within the $tRNA^{Glu}$ and $tRNA^{Lys}$ sequences are needed for targeting the tRNAs for modification, it would be interesting to determine whether the tRNA identity elements uniquely recognized by the cognate aminoacyl-tRNA synthetase also identify the tRNA as a substrate for the modification enzymes. The fact that both $tRNA^{Glu}$ and $tRNA^{Lys}$ are substrates for the modification enzymes allows speculation as to which sequences in the tRNAs are likely to be signals for modification. The 5′ anticodon loop sequence CUUU (32–35) is unique to $tRNA^{Glu}$ and $tRNA^{Lys}$, so it is possible that it could be part of a recognition sequence. It is possible to experimentally address the question of recognition signals for modification using mutant tRNA transcripts, as the availability of the unmodified transcripts provides substrate for assaying the modifying enzymes.

Although it is clear that GluRS is dependent upon the mnm^5s^2U34 modification for efficient aminoacylation of $tRNA^{Glu}$, the complexity of the modification allows the possibility that only part of the modification is required for

aminoacylation. Previous work with tRNA isolated from *E. coli* grown in sulfur-deficient medium indicated that the thio group is nonessential (Agris et al., 1973; Seno et al., 1974). This was confirmed by aminoacylation of $tRNA^{Glu}$ isolated from *asuE* strains of *E. coli* in which thiolation of U34 does not occur (Sullivan et al., 1985). However, in *Clostridium sticklandii* $tRNA^{Glu}$, which contains the selenated derivative of the same modification (mnm^5Se^2U34), deselenation is correlated with a loss of glutamate acceptance in both crude and purified $tRNA^{Glu}$, suggesting that the *C. sticklandii* GluRS is recognizing the selenated part of the modification (Ching & Stadtman, 1982). We are currently addressing this issue by aminoacylation of undermodified $tRNA^{Glu}$ species purified from tRNA modification-deficient strains.

How general are these findings? Yeast tRNAs also contain 2-thiouridine derivatives, e.g., 5-[(methoxycarbonyl)methyl]-2-thiouridine in $tRNA^{Glu}$ (Wong et al., 1979). The fact that antisuppressors in *S. pombe* affect 5-[(methoxycarbonyl)-methyl]-2-thiouridine formation (Grossenbacher et al., 1986; Heyer et al., 1984) is in agreement with the proposition that charging of the undermodified tRNAs may be impaired. Thus, it may be a general phenomenon that the recognition processes ensuring fidelity of translation evolved to employ some of the diverse nucleotide modifications found in tRNA.

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