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Many of the conserved nucleotides of tRNA^{Phe} are not essential for ternary complex formation and peptide elongation

Irina A.Nazarenko, Kristi M.Harrington[1] and Olke C.Uhlenbeck[2]

Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, CO 80309-0215, USA

[1]Present address: University of Colorado Health Sciences Center, 4100 East Ninth Avenue, Denver, CO 80204, USA

[2]Corresponding author

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An RNase protection assay was used to show that the dissociation rate constants and equilibrium constants of unmodified yeast and *Escherichia coli* phenylalanyl-tRNA^{Phe}s to elongation factor Tu from *E.coli* were very similar to each other and to their fully modified counterparts. The affinity of aminoacylated tRNA to elongation factor Tu was substantially lower when GTP analogues were used in place of GTP, emphasizing the importance of the $\beta-\gamma$ phosphate linkage in the function of G-proteins. Fourteen different mutations in conserved and semi-conserved nucleotides of yeast phenylalanyl-tRNA^{Phe} were tested for binding to elongation factor Tu·GTP and assayed for activity in the ribosomal A- and P-sites. Most of the mutations did not severely impair the function of these tRNAs in any of the assays. This suggests that the translational machinery does not form sequence-specific interactions with the conserved nucleotides of tRNA.

Key words: aminoacyl-tRNA/elongation factor Tu/GTP analogues/protein biosynthesis

Introduction

The large number of enzymes that interact with elongator tRNAs can be classified into two broad categories. First, there are enzymes which interact with a specific subset of the tRNA species in the cell. These include the aminoacyl-tRNA synthetases and many of the tRNA modifying enzymes. Second, there are enzymes which must interact with all tRNAs. These include RNase P, elongation factor-Tu (EF-Tu), other tRNA modifying enzymes and, of course, the ribosome. It is likely that these two categories of enzymes achieve their substrate specificity quite differently. In the case of many tRNA synthetases (Pallanck and Schulman, 1992; Giegé *et al.*, 1993) and several modifying enzymes (Bjork, 1992; Lee *et al.*, 1992; Edqvist *et al.*, 1993), it is known that certain specific nucleotides unique to the substrate tRNAs are essential to the specificity. It is often possible to transplant these 'recognition nucleotides' into tRNAs that are not substrates and thereby convert them into substrates (Normanly *et al.*, 1986; Sampson *et al.*, 1989; Schulman and Pelka, 1988; Pallanck and Schulman, 1992). In contrast, enzymes which interact with all tRNAs are likely to recognize their substrates in a different manner. These proteins must either contact functional groups of the limited number of nucleotides present in all tRNAs or interact with regions of the molecule that have a uniform structure. This paper evaluates whether the highly conserved nucleotides in tRNA contribute to the substrate recognition of EF-Tu and the ribosome, two different enzymes in this second category.

The affinity of tRNA for EF-Tu·GTP to form a ternary complex is greatly enhanced by the presence of the esterified amino acid on the 3′-terminus (Jonak *et al.*, 1980; Derwenskus and Sprinzl, 1983). Since certain modifications of the conserved three terminal nucleotides of tRNA inhibit binding to EF-Tu (Ofengand and Chen, 1972; Baksht *et al.*, 1975; Sprinzl *et al.*, 1977), it is likely that these residues contact the protein directly. While short aminoacyl-oligonucleotides can bind EF-Tu (Bhuta and Chladek, 1980; Parlato *et al.*, 1981), their affinity is much lower than that of the intact tRNA, suggesting that additional portions at the tRNA are required for optimal binding. By a combination of footprinting, crosslinking and terminal truncation experiments, the protein is believed to contact the aminoacyl-stem, the T-arm and the variable loop (Jekowsky *et al.*, 1977; Boutorin *et al.*, 1981; Joshi *et al.*, 1984; Wikman *et al.*, 1982, 1987; Rasmussen *et al.*, 1990).

It is likely that the ribosome contacts tRNA more extensively than EF-Tu. Chemical modification and tRNA truncation experiments suggest that the aminoacylated acceptor arm and the anticodon hairpin are the primary contact sites with the ribosome in both the A- and P-sites (Rose *et al.*, 1983; Moazed and Noller, 1990, 1991). However, the successful formation of covalent crosslinks between the 'elbow' of tRNA and certain ribosomal proteins (Lin *et al.*, 1984; Abdurashidova *et al.*, 1991; Mitchell *et al.*, 1993), suggests that this part of the tRNA may contribute to binding as well.

In the present study, we examine how mutations at certain conserved and semiconserved nucleotides of yeast tRNA^{Phe} influence its affinity for EF-Tu, its activity in peptide bond formation and its binding to the ribosomal P-site. Since we have already shown that *Escherichia coli* phenylalanyl-tRNA^{Phe} (Phe-tRNA^{Phe}) lacking all the modified nucleosides is active in these reactions (Harrington *et al.*, 1993), mutant tRNAs prepared by *in vitro* transcription can be used. This work focuses on the conserved and semiconserved nucleotides in the body or 'elbow' region of tRNA. Since many of these residues are involved in the tertiary folding of tRNA^{Phe}, it was important to choose mutants that were minimally impaired in folding so that the sequence could be changed without grossly altering the overall structure.

Table I. Kinetic parameters for interaction with *E.coli* and yeast Phe-tRNA^{Phe} to *E.coli* EF-Tu

	Buffer A			Buffer B		
	K_d (nM)	k_{-1} (s^{-1})	k_1, calc. ($M^{-1}\ s^{-1} \cdot 10^{-5}$)	K_d (nM)	k_{-1} (s^{-1})	k_1, calc. ($M^{-1}\ s^{-1} \cdot 10^{-5}$)
E.coli Phe-tRNA^{Phe}	6	0.0016	2.6	33	0.003	0.9
Yeast Phe-tRNA^{Phe}	4	0.0015	3.7	30	0.003	1.0
Unmodified yeast Phe-tRNA^{Phe}	8	0.0025	3.1	70	0.005	0.7

Results

EF-Tu binding

The binding constants of modified and unmodified yeast Phe-tRNA^{Phe} to *E.coli* EF-Tu were determined using a nuclease protection assay (Louie and Jurnak, 1985). In addition, by determining the dissociation rate constant at the ternary complex, the association rate constant could be calculated. Measurements were carried out in a buffer containing either 25 or 150 mM NH_4Cl. The higher concentration of the ammonium salt is expected to give tighter binding (Harrington *et al.*, 1993) and permits comparison with previous experiments on the interaction between EF-Tu and tRNA (Louie and Jurnak, 1985). The lower salt concentration more closely resembles the buffer used previously for the interaction of ternary complexes with ribosomes (Thompson *et al.*, 1981; Dix *et al.*, 1986).

As has previously been reported (Abrahamson *et al.*, 1985; Louie and Jurnak, 1985), the binding of yeast Phe-tRNA^{Phe} to *E.coli* EF-Tu·GTP is quite similar to that of *E.coli* Phe-tRNA^{Phe}. In the high salt buffer, we obtain a K_d of 4 nM for yeast Phe-tRNA^{Phe} and 6 nM for *E.coli* Phe-tRNA^{Phe} which agrees closely with the values reported by Louie and Jurnak (1985) using the same method and buffer conditions. In the low salt buffer, the values of K_d are higher, but also nearly the same, for the two tRNAs (Table I).

The nucleotide modifications in yeast tRNA^{Phe} have relatively little effect on ternary complex stability in the two buffers tested. In both cases, the unmodified tRNA binds ~2-fold more weakly, presumably as a result of a higher dissociation constant (Table I). A similar result was previously seen for *E.coli* tRNA^{Phe} (Harrington *et al.*, 1993). Taken together, the results indicate that the unmodified yeast tRNA^{Phe} is a suitable background in which to study the role of conserved nucleotides in the interaction of tRNA with *E.coli* EF-Tu.

The stability of the ternary complex is substantially influenced by the chemical structure of the guanidine nucleotides used. The effect of substituting GTP with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), guanosine 5′-*O*-(β,γ-methylenetriphosphate) (GMP-PCP) or guanosine 5′-*O*-(β,γ-imidotriphosphate) (GMP-PNP) using unmodified yeast Phe-tRNA^{Phe} in the high salt buffer is shown in Table II. All three of the analogues resulted in a reduced affinity of Phe-tRNA^{Phe} for EF-Tu. The data in Table II were determined in the presence of 20 μM GTP or GTP analogues, but essentially the same results were obtained when the concentration of nucleotides was either equimolar to the concentration of EF-Tu or increased to 1 mM. This indicates that the higher K_d values for the GTP analogues were not a consequence of undetected contaminating GTP or GDP. The higher K_d values appear to be the result of both an increase in k_{-1} and a decrease in k_1. The same effect of the substitution of GTP for GTP analogues on the K_d of ternary complex was found when modified *E.coli* Phe-tRNA^{Phe} was used as a substrate (data not shown).

Table II. Kinetic parameters for binding of yeast Phe-tRNA^{Phe} transcripts and EF-Tu with GTP and GTP analogues determined in buffer A

	K_d (nM)	k_{-1} (s^{-1})	k_1, calc. ($M^{-1}\ s^{-1} \cdot 10^{-5}$)
GTP	8	0.002	2.50
GTPγS	110	0.006	0.54
GMP-PNP	180	0.010	0.55
GMP-PCP	300	0.012	0.40

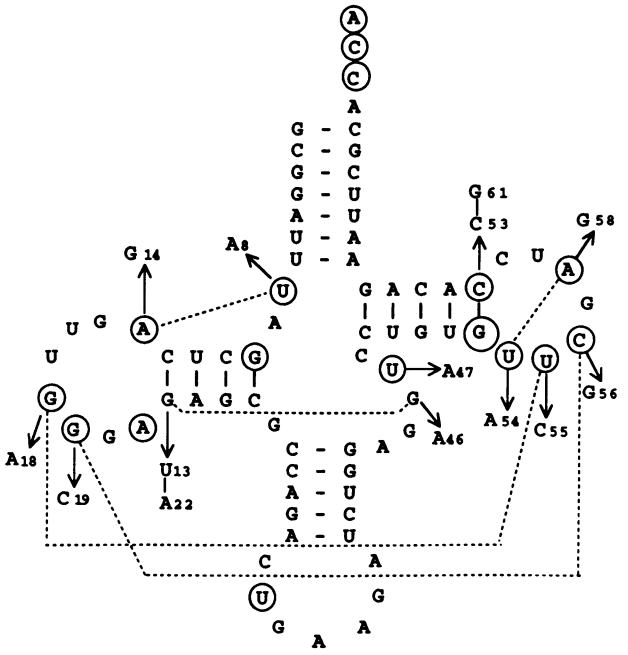

Fig. 1. The tRNA^{Phe} transcript in a cloverleaf structure with tertiary interactions. Conserved nucleotides are circled and substituted nucleotides are indicated by arrows.

Twelve different mutants of yeast tRNA^{Phe} containing changes in 11 of the conserved nucleotides and three of the semiconserved nucleotides in the central core of the tRNA tertiary structure were tested for the binding to EF-Tu (Figure 1). Since our aim was to reveal nucleotides that interact directly with EF-Tu and not simply to show that the folding of tRNA was important for EF-Tu binding, it was important to choose mutations that minimized the change in overall structure. Since nearly all the conserved nucleotides participate in tertiary interactions, this was not always straightforward. In some cases, this could be done.

Table III. Kinetic parameters for ternary complex formation with wild-type and mutated yeast Phe-tRNA^{Phe} transcripts determined in buffer B

Wild-type interaction	Mutation	K_d (nM)	k_{-1} (s^{-1})	k_1, calc. ($M^{-1}\ s^{-1} \cdot 10^{-5}$)	Lead cleavage rel. k_{obs}[a]
–	wild-type	70	0.005	0.7	(1.0)
U54-A58	A58G	245	0.027	1.1	0.26
	U54A	315	0.019	0.6	0.13
G18-U55	U55C	70	0.005	0.7	0.57
	G18A-U55C	245	0.020	0.8	0.42
C13-G22-G46	C13U-G22A-G46A	190	0.019	1.0	0.44
U8-A14	U8A	140	0.013	0.9	0.25
	A14G	175	0.015	0.9	0.30
G19-C56	G19C-C56G	125	0.006	0.5	0.96
	G19C	100	0.010	1.0	0.21
	C56G	140	0.014	1.0	0.19
U47	U47A	120	0.014	1.2	0.59
G53-C61	G53C-C61G	890	0.008	0.09	0.08

[a]Data from Behlen *et al.* (1990).

For example, the semiconserved triplet C13-G22-G46 could be changed to the structurally similar triplet U13-A22-A46 found in other tRNAs, and the highly conserved base pair G19-C56 could be changed to C19-G56 without altering the backbone configuration. However, in other cases (i.e. residues G18, U55, U8 and A14) it was not possible to mutate a nucleotide without potentially affecting folding. In this case, we chose mutations that caused minimal reduction in the rate of cleavage by lead, an assay we have previously shown to be sensitive to the tertiary folding of tRNA^{Phe} (Behlen *et al.*, 1990). By only using tRNA^{Phe} mutants that show significant rates of specific cleavage by lead, it is likely that the mutants retain the overall structure of tRNA^{Phe}.

The equilibrium and rate constants for the interaction of the Phe-tRNA^{Phe} mutants to EF-Tu·GTP are given in Table III. Nearly all the mutated Phe-tRNA^{Phe} transcripts bind to EF-Tu·GTP with K_d values within a factor of 4 of the wild-type tRNA. These include changes known to disrupt partially the tertiary structure such as G19C and C56G. Since a factor of 4 in K_d is in the same range as the differences observed among natural elongator tRNAs (Louie *et al.*, 1984; Louie and Jurnak, 1985) and much less than the difference between initiator and elongator tRNAs (Louie *et al.*, 1984; Seong and RajBhandary, 1987) or between selenocysteinyl-tRNA^{Sec} and seryl-tRNA^{Ser} (Baron and Bock, 1991), we can conclude that most of the conserved and semiconserved nucleotides tested do not contribute significantly to the interaction with EF-Tu. The only mutant that has a large decrease in affinity for EF-Tu is the one that inverts the conserved base pair at the end of the T-stem, G53-C61. However, since this mutant is not able to fold well due to the loss of the hydrogen bond between C61(N4) and P60(O1) (Romby *et al.*, 1987) it is unclear whether the poor binding can be interpreted as the loss of a nucleotide specific contact or as a particularly unsuitable overall structural change.

Binding and activity of mutant tRNA transcripts to ribosomes

It has previously been shown that the unmodified *E.coli* Phe-tRNA^{Phe} transcripts are efficient substrates for the elongation reactions on *E.coli* ribosomes (Harrington *et al.*, 1993). The efficient formation of ternary complexes with most yeast tRNA^{Phe} mutants makes it possible to determine their activity on ribosomes. Before this can be done, however, it is important to evaluate the activity of the unmodified yeast tRNA^{Phe} on the heterologous *E.coli* ribosomes. While successful binding of modified yeast tRNA^{Phe} to the *E.coli* ribosomal A- or P-site has been reported (Lill *et al.*, 1986; Lill and Wintermeyer, 1988), some data have suggested that the binding constant of ternary complex containing yeast Phe-tRNA^{Phe} to the *E.coli* ribosomal A-site may be as much as 10^3-fold weaker than the ternary complex with *E.coli* Phe-tRNA^{Phe} (Dix *et al.*, 1986). In addition, it is possible that the nucleotide modifications may contribute more to the activity of yeast tRNA^{Phe} than they do to *E.coli* tRNA^{Phe}.

Preliminary filter binding experiments where poly(U)-programmed 70S ribosomes with an occupied P-site were mixed with *E.coli* or yeast tRNA^{Phe} ternary complexes containing GMP-PCP revealed little difference in the ability of the two tRNAs to promote binding to the ribosomal A-site. A similar result was obtained with GTPγS. This prompted an ultracentrifugation assay (Dix *et al.*, 1986) where GMP-PCP ternary complexes formed with the aminoacylated yeast and *E.coli* tRNA transcripts were titrated with varying concentrations of programmed ribosomes. As shown in Figure 2, relatively little difference between the two ternary complexes was observed. These curves closely resemble the curve for the yeast Phe-tRNA^{Phe} transcript determined previously (Dix *et al.*, 1986). We do not think it is possible to estimate reliably an affinity constant using these data because of the low affinity of aminoacyl-(aa-)tRNA for EF-Tu in the presence of GMP-PCP. Nevertheless it is clear that the ternary complex made with unmodified yeast Phe-tRNA^{Phe} is a good substrate for *E.coli* ribosomes and can be used as a background for the mutagenesis experiments.

To evaluate the activity of yeast tRNA^{Phe} mutants on ribosomes, ternary complexes were prepared with [^{3}H]Phe-tRNA^{Phe} and [γ-^{32}P]GTP, mixed with programmed ribosomes with occupied P-sites and the amount of GTP hydrolysed and dipeptide formed was determined after 1 min. The results for each mutant (Table IV) are normalized to the corresponding value for the wild-type tRNA transcript. Despite the substitution of conserved or semiconserved

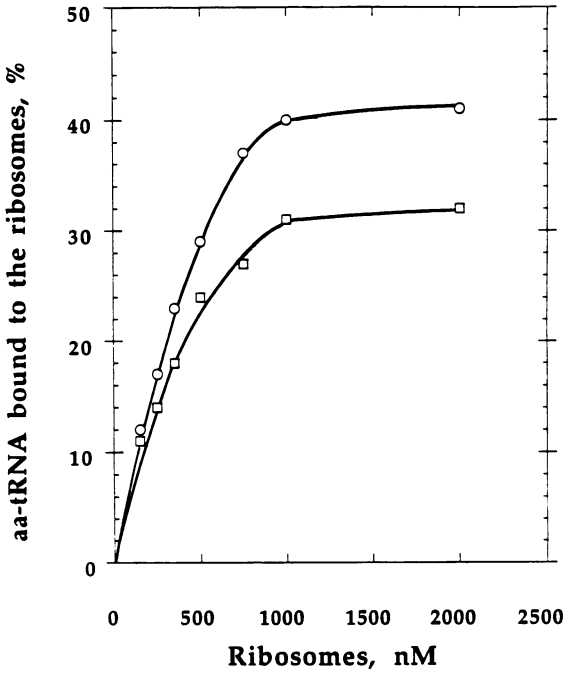

Fig. 2. Enzymatic binding of *E.coli* (□) and yeast (○) Phe-tRNA^{Phe} transcripts to *E.coli* ribosomes. Binding of [^{3}H]Phe-tRNA^{Phe} to the ribosomes at different concentrations was measured by ultracentrifugation as described in Materials and methods.

Table IV. The extent of the GTP-hydrolysis and peptide formation on poly(U)-programmed ribosomes with mutated yeast Phe-tRNA^{Phe} transcripts determined in buffer B

Wild-type interaction	Mutation	GTP hydrolysis (rel.)	Dipeptide formation (rel.)
–	wild-type	(1.0)	(1.0)
U54-A58	A58G	0.72	0.73
	U54A	0.48	0.39
G18-U55	U55C	1.00	1.30
C13-G22-G46	C13U-G22A-G46A	1.10	0.94
U8-A14	U8A	0.83	0.90
	A14G	0.84	0.66
G19-C56	G19C-C56G	0.90	0.85
	G19C	1.00	1.00
U47	U47A	1.10	1.00
G53-C61	G53C-C61G	0.20	0.15

nucleosides, all but one of the mutants are able to promote the elongation reaction on the ribosomes at least 40% as well as the wild-type transcript. While the results in Table IV were obtained in a buffer containing 10 mM Mg^{2+}, experiments were also performed in the 5 mM $MgCl_2$ buffer previously used to characterize the elongation reaction (Dix *et al.*, 1986; Thompson *et al.*, 1981, 1986). Though the extent of GTP hydrolysis and dipeptide formation with both wild-type and mutant transcripts was lower, the relative extents were quite similar to the data in Table IV (data not shown). Since the effect of most of the mutations is either modest or non-existent in the assay, we can conclude that the conserved nucleotides tested contribute little, if any, to the function of tRNA on ribosomes.

Table V. Rate constants for the GTP-hydrolysis and peptide formation with yeast Phe-tRNA^{Phe} transcript and C53-G61 mutant determined in buffer B

	k_{GTP} (M^{-1} s^{-1})	k_{pep} (s^{-1})
Yeast transcript	12.4×10^7	5.2
G53C-C61G	5.0×10^6	2.0

Table VI. Non-enzymatic binding of mutated yeast Phe-tRNA^{Phe} transcripts to the *E.coli* ribosomes determined in buffer B

Wild-type interaction	Mutation	[^{3}H]Phe-tRNA^{Phe} binding (rel.)
–	wild-type	(1.0)[a]
U54-A58	A58G	0.9
	U54A	0.6
G18-U55	U55C	1.2
C13-G22-G46	C13U-G22A-G46A	1.0
U8-A14	U8A	0.9
	A14G	0.8
U47	U47A	0.8
G53-C61	G53C-C61G	0.7

[a]Binding of wild-type tRNA was 40% of tRNA input.

The mutation which inverted the G53-C61 base pair at the end of the T-stem had the greatest effect at GTP hydrolysis and dipeptide formation. While this might suggest that one of these conserved residues interacts with the ribosome directly, it was possible that this low extent of reaction was due to the weak affinity of this mutant for EF-Tu, resulting in incomplete ternary complex formation. In order to investigate this possibility further, a kinetic analysis of the reaction with the C53-G61 mutant was carried out so that the rate constants of GTP hydrolysis (k_{GTP}) and peptide formation (k_{PEP}) could be obtained. As shown in Table V, both rate constants were ~2.5-fold slower for the mutant than for the wild-type control. The relatively small effect is of the same order as the contribution of nucleotide modifications to these complex rate constants (Harrington *et al.*, 1993) and suggests that these residues are also unlikely to be critical for tRNA binding on ribosomes.

It is known that when aa-tRNA is incubated at 37°C with programmed ribosomes having an empty P-site in the absence of EF-Tu, most of the aa-tRNA is found in the P-site (Rheinberger *et al.*, 1983). It was later demonstrated that under these conditions aa-tRNA first interacts with the A-site and then enters the P-site (Schilling-Bartezko *et al.*, 1992). We used poly(U)-programmed ribosomes with unoccupied P-sites to test the affinity of mutated Phe-tRNA^{Phe} transcripts. The results presented in Table VI show that all the mutants tested are at least 60% active in non-enzymatic binding to ribosomes. In this case, the Phe-tRNA^{Phe} transcript with the G53-C61 to C53-G61 mutant does not differ from the other mutant transcripts.

Discussion

The results presented here and previously published data (Harrington *et al.*, 1993) indicate that modifications in both yeast and *E.coli* Phe-tRNA^{Phe} do not greatly affect their binding to *E.coli* EF-Tu. Aminoacylated transcripts of both tRNAs lacking modified nucleosides demonstrated only a

1.5- to 2-fold difference in the equilibrium dissociation constants relative to their natural counterparts. This difference was shown to be mostly the result of an increase in the dissociation rate (k_{-1}) of unmodified aa-tRNA from EF-Tu. Nevertheless, as stated previously (Harrington *et al.*, 1993), the small effect on k_{-1} may have large consequences in the overall accuracy of translation. Since EF-Tu is thought to bind primarily to the acceptor stem and the T-hairpin of the tRNA molecule, the results presented here suggest that the modified nucleosides in this region (s^4U8, D16, D17, D20, m_2^2G26, m^7G46, X47, m^5C49, T54, Ψ55 and m^1A58) do not contact EF-Tu directly and their removal does not perturb the structure of tRNA in such a way as to disrupt the interaction of tRNA with EF-Tu.

The tertiary structure of *E.coli* $tRNA^{Phe}$ is believed to resemble yeast $tRNA^{Phe}$ closely. Both molecules have the same number of nucleotides and share seven of nine tertiary interactions. The U20G mutation of *E.coli* $tRNA^{Phe}$ makes it a good substrate for yeast FRS and the U60C mutation makes it closely resemble yeast $tRNA^{Phe}$ in its susceptibility to specific cleavage by Pb^{2+} (Sampson *et al.*, 1989; Behlen *et al.*, 1990). It is therefore not surprising that both tRNAs bind *E.coli* EF-Tu with very similar affinities, both as modified tRNAs (Abrahamson *et al.*, 1985; Louie and Jurnak, 1985) and as unmodified tRNAs. This means it should be possible to use mutations in either molecule to study their effect on EF-Tu binding. Here we have used heterologous yeast transcripts because many more mutants are available in this background and more information is available on the folding of these mutants (Behlen *et al.*, 1990). In addition, *E.coli* transcripts are prone to form alternative conformations (Peterson and Uhlenbeck, 1992) which makes it difficult to interpret binding data.

Prompted by the fact that removal of the γ-phosphate from GTP reduces the affinity of aa-tRNA to EF-Tu by about a factor 100 (Pingoud *et al.*, 1982), we have investigated whether different GTP analogues have a similar effect. Previous reports (Arai *et al.*, 1974; Moazed *et al.*, 1988; Delaria *et al.*, 1991) noted lower stability of ternary complexes formed by GMP-PCP. In the present more quantitative study, it was found that all three GTP analogues substantially reduced the affinity of aa-tRNA for EF-Tu. Binding was 43-, 23- or 15-fold weaker when GMP-PCP, GMP-PNP or GTPγS respectively was used in place of GTP.

The poor binding of aa-tRNA in the presence of GTP analogues can be understood from the structure of EF-Tu (Berchtold *et al.*, 1993) and the general mechanism of action of G-proteins. The precise positioning of γ-phosphate of GTP is critical to organize the structure of the protein in the neighbourhood of the tRNA binding site. In the ternary complex, numerous hydrogen bonds are believed to form between β- and γ-phosphate oxygens and different parts of the protein. When the covalent β−γ bond is cleaved, the structure disassembles and aa-tRNA is released. Presumably when GTP analogues are used, the overall stability of the complex is reduced because an identical hydrogen bonding pattern cannot form. This could be due either to the altered β−γ dihedral angle in the case of GMP-PCP (Yount *et al.*, 1971) or the higher pK_as of the γ-phosphate in all three derivatives (Irani and Callis, 1961; Yount *et al.*, 1971). In addition, the introduction of a sulfur in place of oxygen in GTPγS, could alter the hydrogen bond to one of the crucial oxygens. Thus, even though the binding of the GMP portion may be similar, the assembly of the critical portion of the structure is incorrect, leading to weaker tRNA binding.

The lower stability of the ternary complexes containing GTP analogues greatly complicates their use in quantitative protein synthesis experiments from both a practical and theoretical standpoint. Since ternary complexes containing GTP analogues are not fully saturated with tRNA at concentrations lower than 500−1500 nM (depending on the analogue and buffer used), experiments using lower concentrations must at least account for the reduced amount of active species. In addition, since GTP analogues intrinsically reduce the affinity of EF-Tu for aa-tRNA, it is quite possible that they will intrinsically reduce the affinity of ternary complexes to ribosomes as well. A careful analysis of this complex coupled equilibrium is clearly required.

In this report, we have determined the affinities of 14 different mutations in conserved and semiconserved nucleotides of yeast $tRNA^{Phe}$ to *E.coli* EF-Tu·GTP. The mutations were chosen to change the residue but, as far as possible, maintain the tRNA tertiary structure. In nearly every case, the effects of the mutations were very small. K_d values were increased by less than 4-fold, primarily as a result of an increased dissociation rate. The small differences between the mutants are not easily interpreted. There is a rough correlation between the increase in K_d and the reduction in Pb^{2+} cleavage rate. This may suggest a requirement for the correct folding of tRNA for optimal EF-Tu binding. However, other tRNA mutants known to misfold (G19C) appear to bind EF-Tu almost normally. In any case, the effect of most of the mutations on the free energy of binding to EF-Tu was very small (0.8 Kcal/mol) relative to the total free energy of binding (−9.1 Kcal/mol) and the variation among mutants was in the same range as seen among natural tRNAs of different specificities (Louie *et al.*, 1984; Louie and Jurnak, 1985). This strongly suggests that interactions with many of the tRNA conserved nucleotides contribute very little to the free energy of EF-Tu binding. Presumably, EF-Tu derives much of its binding energy with the body of tRNA through interaction with the folded ribose phosphate backbone.

The tRNA mutation with the largest effect on K_d with EF-Tu is the transversion of the highly conserved base pair G53-C61 at the end of the T-stem to C53-G61. Interestingly, this mutation shows not only a 1.5-fold increase in the dissociation rate, but also an 8-fold increase in the association rate. The effect on the dissociation rate is consistent with results from both footprinting (Boutorin *et al.*, 1981) and crosslinking (Wikman *et al.*, 1987) experiments suggesting that this part of the tRNA interacts with EF-Tu. However, the tertiary structure of the C53-G61 tRNA is quite different from the native structure (Romby *et al.*, 1987; Behlen *et al.*, 1990), so it is not possible to conclude that the effect on binding is the result of a disruption of a direct contact with the protein. The effect of the C53-G61 mutation on the association rate is difficult to explain. Louie and Jurnak (1985) noted that the association rate for ternary complex formation is unexpectedly slow (10^5 to 10^6 M^{-1} s^{-1}) and appears to vary substantially among different tRNAs. As noted above, several of the GTP analogues also reduce the association rate. These facts are consistent with the suggestion that binding may be partially rate-limited by a conformational rearrangement (Weygand-Durasevic *et al.*, 1981; Adkins *et al.*, 1983; Hazlett *et al.*, 1989).

A preliminary survey of the effects of the different tRNA mutations on dipeptide synthesis activity and ribosomal P-site binding has also been completed. In many respects, the results are quite similar to those on ternary complex formation. In most cases, the ternary complexes containing mutant tRNAs are able to bind poly(U)-programmed ribosomes and make dipeptide in an amount comparable to the wild-type tRNA. The only substantial decrease in the extent of the elongation reactions was observed for the G53-C61 to C53-G61 mutation. However, the rates of GTP hydrolysis and dipeptide formation for this mutant are essentially the same as for the wild-type transcript. It is likely that the lower extent of synthesis is simply due to the weaker binding of this mutant to EF-Tu, resulting in a much lower concentration of active ternary complex. Thus, there is no indication that any of the tRNA mutants are impaired in dipeptide synthesis. Similarly, none of the tRNA mutants show greatly reduced binding to the ribosomal P-site.

While it is not clear what decrease in activity should be considered significant for tRNA–ribosome interactions, the limited amount of data presented here suggest that, like EF-Tu, the free energy interaction of tRNA with ribosomes may be dominated by contacts formed with the folded ribosome–phosphate backbone and not by contacts with the highly conserved nucleotides. Since this conclusion is in conflict with a number of models suggesting sequence-specific interactions between conserved tRNA nucleotides and ribosomes (Ofengand and Henes, 1969; Erdmann *et al.*, 1973; Sprinzl *et al.*, 1976; Lin *et al.*, 1984; Helk and Sprinzl, 1985; Abdurashidova *et al.*, 1991; Mitchell *et al.*, 1993), it is important to measure the activity of these mutant tRNAs in other biochemical assays that evaluate different steps of the translation process. It is possible that mutation of conserved residues may have much larger effects in, for example, translocation or tRNA release from the ribosome.

If mutations in the tRNA conserved nucleotides have relatively little effect in biochemical assays of translation, why are these residues so highly conserved? It is possible that one or more other steps in tRNA metabolism provide the selective pressure for the conserved nucleotides. Indeed, several of the same mutations studied in this work have been found to have substantial effects in other steps of tRNA metabolism including tRNA modification (Drabkin and RajBhandary, 1985; Edqvist *et al.*, 1993), RNase P activity (Bear *et al.*, 1988; Thurlow *et al.*, 1991) and tRNA splicing (Greer *et al.*, 1987; Mattocia *et al.*, 1988; Reyes and Abelson, 1988). Another possibility is that most of the selective pressure for the conserved sequences does indeed come from the ribosome, but the biochemical effects are quite small. It is known that the tRNA modifications cause relatively small effects on individual rate constants in the translational mechanism, but they substantially improve the overall accuracy of translation (Harrington *et al.*, 1993). It may be that having a proper set of conserved nucleotides in tRNA may only alter the translational rate or accuracy a small amount, but be important for the success of the cell. Thus, the relationship between the degree of phylogenetic conservation and the magnitude of a biochemical effect is likely to be complex. An interesting experimental approach towards resolving this issue is to use *in vitro* evolution methods to obtain tRNAs with altered tertiary interactions (Peterson *et al.*, 1993) and evaluate their function in translation, both biochemically and genetically.

Materials and methods

Preparation of aminoacylated tRNAs

Wild-type and mutant yeast $tRNA^{Phe}$s were prepared by transcription of the corresponding plasmid DNA by T7 RNA polymerase in the presence of an excess of 5′-GMP to ensure 5′-monophosphate termini (Sampson and Uhlenbeck, 1988). tRNA transcripts were purified on denaturing polyacrylamide gels and stored in deionized water. Prior to aminoacylation, the tRNA samples were heated at 85°C for 2 min in water and slow cooled to 25°C. Aminoacylation reactions were carried out in 80 μl reaction mixtures, containing 30 mM HEPES–KOH, pH 7.45, 2 mM ATP, 10 mM $MgCl_2$, 25 mM KCl, 4 mM dithiothreitol, 5 μM tRNA, 25 μM [^{3}H]phenylalanine (45–72 Ci/mmol, Amersham) and 50 nM yeast Phe-tRNA synthetase for 15 min at 37°C. [^{3}H]Phe-$tRNA^{Phe}$ was purified by applying the reaction mixture to a 40 μl TSK Fractagel DEAE column equilibrated in 50 mM NaCl, 50 mM sodium acetate, pH 7.0, washing with three 150 μl aliquots of 150 mM NaCl in the same buffer and eluting with 100 μl of 600 mM NaCl in the same buffer (Harrington *et al.*, 1993). The modified yeast $tRNA^{Phe}$ (Boehringer-Mannheim) was aminoacylated and purified by the same protocols.

Binding of aminoacylated tRNA to EF-Tu

Escherichia coli EF-Tu (a gift of F.Jurnak, University of California-Riverside) was prepared free of nucleotides as previously described (Thompson *et al.*, 1981). To prepare the binary complex, 1 μM EF-Tu was incubated at 37°C for 15 min with 3 mM phosphoenolpyruvate, 10 U/ml pyruvate kinase (Sigma) and 20 μM GTP in buffer A (50 mM Tris–HCl, pH 7.4, 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol) or buffer B (same as A, but 25 mM NH_4Cl). The equilibrium dissociation constants for ternary complex were determined by an RNase protection assay as described by Louie and Jurnak (1985) with some minor modifications. 100 μl reactions containing varying concentrations (10–500 nM) of EF-Tu·GTP and varying concentrations (2–200 nM) of appropriate [^{3}H]Phe-$tRNA^{Phe}$ in one of the above regeneration buffers were incubated for 15 min at 4°C to allow ternary complex to form. Then 5 μl of 2 mg/ml pancreatic RNase (Sigma) was added and the incubation continued for 15 s to degrade unbound [^{3}H]Phe-$tRNA^{Phe}$. Each reaction was terminated by the addition of 50 μl of 1 mg/ml unfractionated yeast tRNA (Sigma) and 200 μl of cold 10% TCA. Radioactive precipitates were collected on nitrocellulose filters and counted. Control reactions containing either a large excess of tRNA or EF-Tu allowed the fraction of active EF-Tu and the fraction of active [^{3}H]Phe-$tRNA^{Phe}$ to be determined and thus permitted the value of K_d to be calculated.

The K_d values for ternary complexes formed with GTP analogues were determined with a slightly different protocol. In this case, 1 μM nucleotide-free EF-Tu was incubated with 20 μM GTP or GTP analogue in buffer A without the regeneration system to form binary complex. Varying concentrations of this binary complex were mixed with varying [^{3}H]Phe-$tRNA^{Phe}$ concentrations in buffer A containing 20 μM GTP or GTP analogue and K_d was determined as before. Although all three GTP analogues contained less than 1% nucleotide contaminants as judged by thin layer chromatography, two control experiments were performed to address the possibility that impurities were responsible for the altered K_d values. In one, the nucleotide concentrations were increased to 1 mM throughout. In the other, equimolar (1 μM) GTP or GTP analogue was used to prepare binary complex and no nucleotide was present in the tRNA binding buffer.

Dissociation rate constants were determined by the RNase protection assay in 100 μl reactions by preparing ternary complex with 0.1 μM [^{3}H]Phe-$tRNA^{Phe}$ and 1 μM EF-Tu in the presence of regeneration system as described above. After the addition of 5 μl of 2 mg/ml RNase, 10 μl aliquots were withdrawn at appropriate times (0.3–4 min), terminated and analysed as described above. A semilogarithmic plot of the fraction of tRNA bound to EF-Tu versus time yields the first order rate constant for the dissociation of aa-tRNA from EF-Tu.

Binding of the ternary complex to the ribosomes

Poly(U)-programmed 70S ribosomes containing *N*-acetyl-[^{14}C]Phe-$tRNA^{Phe}$ in the P-site were prepared in buffer B as previously described (Thompson *et al.*, 1981). The fraction of active poly(U)-programmed ribosomes was determined from the end-point of GTP hydrolysis and peptide formation in the presence of excess of *E.coli* Phe-$tRNA^{Phe}$·EF-Tu·GTP and the active concentration of ternary complexes was determined using an excess of ribosomes. For all the experiments described below, controls performed without ribosomes were subtracted from the values of tRNA bound, GTP hydrolysed and peptide formed.

For the ribosome binding experiments, 0.1 μM ternary complex was

formed of nucleotide-free EF-Tu, [^{3}H]Phe-tRNA^{Phe} and GMP-PCP instead of GTP to prevent GTP hydrolysis. After 15 min at 4°C, the complex was added to varying concentrations of poly(U)-programmed ribosomes in 50 μl of buffer B. Fraction of ternary complex bound to the ribosomes was determined by ultracentrifugation technique as described before (Dix *et al.*, 1986).

Non-enzymatic binding of Phe-tRNA^{Phe} to the ribosomes

25 pmol of poly(U)-programmed ribosomes with unoccupied P-site were incubated with 5 pmol of [^{3}H]Phe-tRNA^{Phe} in 25 μl of buffer B at 37°C for 10 min. Fraction of [^{3}H]Phe-tRNA^{Phe} bound to the ribosomes was determined by filtration through the nitrocellulose filters (Schilling-Bartezko *et al.*, 1992).

Single turnover elongation reactions on the ribosomes

Ternary complexes were formed with 2.5 pmol of [^{3}H]Phe-tRNA^{Phe} (45-60 Ci/mmol), 2.5 pmol of [γ-^{32}P]GTP (40–60 Ci/mmol) and 5 pmol of *E.coli* EF-Tu free of nucleotides in 5 μl of buffer B. After 15 min at 4°C, the reaction was added to 20 pmol of poly(U)-programmed 70S ribosomes made with 20 pmol *N*-acetyl [^{14}C]Phe-tRNA^{Phe} (50 mCi/mmol) in the P-site in 20 μl of buffer B. To determine the extent of the reactions, the incubation was terminated after 1 min by the addition of 10 μl of 0.5 M EDTA, and 12 μl aliquots were analysed for GTP hydrolysis and *N*-acetyl-[^{14}C]phenylalanyl-[^{3}H]phenylalanine formation by previously described procedures (Eccleston *et al.*, 1985; Thompson *et al.*, 1986). To determine the rate constants for GTP hydrolysis (k_{GTP}) and dipeptide formation (k_{PEP}), the above reaction was performed in a rapid mixing apparatus (Eccleston *et al.*, 1980) where the incubation was terminated at times between 0.1 and 30 s. Rate constants were obtained by computer simulation of the reaction progress curves (Thompson *et al.*, 1980) taking into account the concentrations of active ribosomes and ternary complex.

Each rate constant reported here is a result of three determinations with the variation in absolute values up to ±10%, depending on the preparation of EF-Tu and ribosomes used. The experiments comparing tRNA mutants were always performed side by side with the same preparation of EF-Tu and ribosomes.

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