Determination of Recognition Nucleotides for *Escherichia coli* Phenylalanyl-tRNA Synthetase†

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ABSTRACT: The nucleotides in *Escherichia coli* $tRNA^{Phe}$ required for recognition by its cognate synthetase have been determined in vitro by measuring the kinetic parameters for aminoacylation using mutant $tRNA^{Phe}$ transcripts with purified *E. coli* $tRNA^{Phe}$ synthetase. The substitution of 11 nucleotides in *E. coli* $tRNA^{Phe}$ is shown to decrease the k_{cat}/K_M by as much as 1000-fold relative to the wild type. The most important recognition elements are the three anticodon nucleotides G34, A35, and A36. The recognition set also includes nucleotides in the variable pocket (U20 and U59), the acceptor end (A73), and the tRNA central core (G10, C25, A26, G44, and U45). Many of the recognition nucleotides are also among the residues comprising the identity set determined in vivo using an amber suppressor $tRNA^{Phe}$ [McClain, W. H., & Foss, K. (1988) *J. Mol. Biol. 202*, 697–709]. As could be anticipated from the very different methods used, some nucleotides in the identity set determined by the suppressor method were not among the recognition nucleotides and vice versa. The *E. coli* $tRNA^{Phe}$ recognition data can also be compared to the recognition sets for yeast and human $tRNA^{Phe}$ determined previously. The results indicate that the mechanism by which phenylalanyl-tRNA synthetases recognize their substrates seems to have diverged somewhat among different species. For example, nucleotide 20 in the D-loop, the anticodon nucleotides and the discriminator base 73 are important for the recognition by all three enzymes. However, recognition of the tRNA central core nucleotides is unique to *E. coli* FRS. In the course of our study, we found several point mutations that disrupted the tRNA structure in an unpredictable fashion, making it essential to perform independent folding assays to validly interpret the activity of a mutant tRNA.

The correct selection and aminoacylation of a tRNA by its cognate synthetase is a crucial step for accurate protein synthesis. It is believed that synthetases are able to discriminate between tRNAs by forming specific contacts with a limited number of nonconserved nucleotides dispersed throughout the cognate tRNA as well as sensing relatively subtle differences in the spatial arrangement of the sugar–phosphate backbone of different tRNAs (Normanly et al., 1986b; Schimmel, 1987; Hou & Schimmel, 1988; McClain & Foss, 1988b; Schulman & Pelka, 1988; Himeno et al., 1989; Rould et al., 1989; Sampson et al., 1990; Perret et al., 1990; Schulman, 1991). tRNA nucleotides required to ensure correct aminoacylation by its cognate synthetase have been identified in two quite different ways. An in vivo method evaluates mutant amber suppressor tRNAs for their efficiency of suppression and the identity of the inserted amino acid (Normanly et al., 1986a; McClain & Foss, 1988c; Rogers & Söll, 1988). The resulting "identity set" is defined as the nucleotides that must be present in the suppressor tRNA for the efficient insertion of the correct amino acid in suppressed protein. A second, in vitro, method involves preparing tRNA mutants by in vitro transcription and measuring their aminoacylation kinetics with purified aminoacyl-tRNA synthetase (Sampson & Uhlenbeck, 1988; Schulman & Pelka, 1988; Francklyn & Schimmel, 1989; Himeno et al., 1990). The resulting "recognition set" is defined as those nucleotides that, upon mutation, substantially reduce the catalytic efficiency of aminoacylation but do not alter tRNA folding. Both methods have revealed that relatively few nucleotides in a given tRNA are critical in the aminoacylation process. However, it is clear that the two methods will not necessarily reveal precisely the same nucleotides due to the very different circumstances under which aminoacylation occurs. The in vitro method, while measuring only aminoacylation, employs nonphysiological enzyme, substrate, and salt concentrations which can potentially result in a different rate-limiting step in the reaction. In the in vivo method, the other tRNA synthetases can potentially compete for a mutant substrate, resulting in an identity set that reflects both the elements within the tRNA that are required for a productive interaction with its cognate synthetase and also those preventing interactions with noncognate synthetases. However, the success of a particular tRNA mutant in the suppression assay in vivo is not always indicative of a fully active tRNA substrate in vitro (Hou & Schimmel, 1989; results here), and its failure may not be the result of poor aminoacylation but rather a defect in tRNA maturation.

This work analyzes the in vitro aminoacylation properties of mutant *Escherichia coli* $tRNA^{Phe}$ transcripts with purified *E. coli* phenylalanyl-tRNA synthetase (FRS)[1] to determine the recognition set for this system. Since a fairly complete determination of the identity set of the *E. coli* $tRNA^{Phe}$ amber suppressor has been reported (McClain & Foss, 1988a), this work permits a comparison of the two very different methods of locating nucleotides important for aminoacylation. In addition, the existing data for the yeast and human FRS–$tRNA^{Phe}$ interaction (Sampson et al., 1989; Nazarenko et al., 1992) allow a comparison of FRS recognition sets between prokaryotes and eukaryotes.

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[1] Abbreviations: TCA, trichloroacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FRS, phenylalanyl-tRNA synthetase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NTP, nucleoside triphosphate; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Construction of Plasmids. All the T7 transcription templates were cloned except two, whose constructions are described below. The plasmids containing variant tRNA genes were constructed by cloning six synthetic DNA oligonucleotides into pUS618 as described by Sampson and Uhlenbeck (1988). The plasmids containing a U60C substitution (to measure lead cleavage) were constructed by subcloning the 400 base pair C60 *Sty*I/*Aat*II fragment from the C60 mutant plasmid into the tRNA plasmid of interest. All sequences of cloned tRNA genes were confirmed by the dideoxy sequencing method of double-stranded plasmid DNA using Sequenase (United States Biochemical; Sanger et al., 1977).

Construction of the G10A,C25U,U60C Mutant. Because it proved difficult to prepare by subcloning, the tRNA gene containing the mutations G10A,C25U,U60C was prepared by PCR amplification of the plasmid harboring the G10A,-C25U mutation with an upstream 20-nucleotide primer containing the T7 promoter (Sampson & Uhlenbeck, 1988) and a downstream primer complementary to the last 31 nucleotides of the *E. coli* tRNA^{Phe} sequence with the appropriate A to G substitution (5′TGGTGCGCG-GACTCGGGATCGAACCAAGGAC3′). Wild-type tRNA^{Phe} with a U60C mutation generated from transcription of a PCR template was found to have a similar rate of lead cleavage to tRNA^{Phe} U60C prepared from plasmid DNA.

Construction of the Yeast tRNA^{Phe} G20U,G26A, A44G,G45U Mutant. As an alternative to cloning, the yeast tRNA^{Phe} quadruple mutant was prepared by transcription of synthetic *DNA* oligonucleotides. Two complementary oligonucleotides with the appropriate substitutions were prepared: the first was a 57-mer which containing the T7 promoter followed by the tRNA^{Phe} gene sequence from nucleotides 1 to 40, and the second was a 48-mer which contained the complementary strand of the tRNA gene from residues 29 to 76. The oligonucleotides were annealed through their 12 complementary residues. This template was elongated in a reaction containing 1.3 μM annealed DNA, 0.4 mM each dNTPs, 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 10 mM DTT, and 600 units of Moloney murine leukemia virus reverse transcriptase (United States Biochemical) for 1 h at 37 °C. The resulting double-stranded 93-nucleotide template was phenol-extracted, ethanol-precipitated, and used as a substrate for in vitro transcription as described below.

Transcription of tRNA. Transcription templates derived from PCR-amplified DNA, reverse transcriptase-extended DNA, or *Bst*NI-digested plasmid DNA were used to generate runoff transcription products in vitro with T7 RNA polymerase as described previously (Sampson & Uhlenbeck, 1988). tRNA with a 5′-monophosphate was obtained by priming transcription with a 5-fold excess of GMP over GTP. Internally ^{32}P-labeled transcripts for lead cleavage were prepared as described by Behlen et al. (1990). The RNA was purified to single-nucleotide resolution on a 15% polyacrylamide gel to ensure the presence of a 3′-terminal A residue. The RNAs were excised from the gel, eluted, and purified from residual EDTA over a Fractagel/DEAE column, ethanol-precipitated, and resuspended in water (Behlen et al., 1990; Sampson et al., 1990). The concentration of unlabeled RNA was determined assuming an extinction coefficient of 5.0×10^5 M^{-1} cm^{-1} at 260 nm. The kinetics of tRNA cleavage with lead were carried out on radiolabeled tRNA as described by Behlen et al. (1990). All *E. coli* tRNA^{Phe} mutants that were tested for lead cleavage contain U60C.

Modified E. coli tRNA^{Phe} and Amber Suppressor tRNA^{Phe}. The natural tRNA^{Phe} isolated from *E. coli* was obtained from Subriden RNA (Rollingbay, WA). The *E. coli* tRNA^{Phe} amber suppressor gene contained on a plasmid (pGFIB:Phe) was kindly provided by Dr. J. Abelson (California Institute of Technology, Pasadena, CA). The tRNA^{Phe} suppressor overproduced by the plasmid in XAC-1 cell was isolated as described by Hou and Schimmel (1988). The resulting tRNA preparation contained only small amounts of host tRNA^{Phe} (which comigrates on acrylamide gels) as judged by analysis of tRNA isolated from the XAC-1 cells without the plasmid.

Purification of E. coli Phenylalanyl-tRNA Synthetase. The synthetase was purified from KMBL1164 cells containing the cloned synthetase genes on the plasmid pBI (Plumbridge et al., 1980) provided by Dr. M. Springer (Institut de Biologie Physico-Chemique, Paris, France). The purification takes advantage of the large sedimentation coefficient (8.6 S) of the synthetase (Stulberg, 1967). All purification steps were carried out at 4 °C. Cells from a 1.5-L culture grown in LB broth were lysed in 60 mL of 20 mM Tris-HCl, pH 8.0, 50 mM NH_4Cl, 3 mM DTT, 1 mM EDTA, 5% glycerol, and 0.5 mg/mL lysozyme. After lysis, the cells were centrifuged at 18000*g* for 50 min, and the supernatant was brought to 15 mM $MgCl_2$ and centrifuged for 1.5 h at 125000*g* to remove the ribosomes. The supernatant from this centrifugation was subjected to an additional centrifugation at 125000*g* for 15 h. The pellet was resuspended in 1.0 mL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, and 5 mM β-mercaptoethanol. The sample was then applied to a 1-mL FPLC Mono Q (anion-exchange) column and eluted with a linear 24-mL gradient of 40–400 mM KCl in the above buffer. The synthetase activity was found to elute at 200–250 mM KCl. SDS gels of the peak fractions were found to contain the two subunits of FRS at approximately 80% purity. The protein can be stored at –20 °C in 50% glycerol without significant loss of activity over several months. More than 2 mg of protein per liter of cells was obtained; the protein concentration was estimated using the Bradford method (1976) against BSA as the standard. Using 1 unit equals 1 nmol tRNA^{Phe} aminoacylated/min at 37 °C, the specific activity was found to be 290 units/mg, which is comparable to values obtained by other purification methods (Conway et al., 1962; Stulberg, 1967; Hanke et al., 1975).

Aminoacylation of tRNAs. Aminoacylation kinetics were carried out at 37 °C in 60-μL reactions containing 30 mM HEPES, pH 7.45, 25 mM KCl, 15 mM $MgCl_2$, 4 mM DTT, 2 mM ATP, 10 μM ^{3}H-labeled phenylalanine, and 0.4–12 nM (0.03–1.0 unit/mL) *E. coli* phenylalanyl-tRNA synthetase. RNA concentrations ranged from 25 nM to 4 μM. To obtain fully active transcripts after storage at –20 °C, the tRNA was heated to 90 °C in water, slowly cooled to room temperature, and then preincubated at 37 °C for 3–5 min in the reaction buffer prior to initiation by the addition of synthetase. At 15–55-s time points, aliquots were removed and spotted on Whatman 3MM filter paper that had been previously soaked in 10% TCA and dried. The filter papers were then washed 4 times in 5% TCA and dried, and the [^{3}H]phenylalanine was determined by scintillation counting in toluene/PPO. Each tRNA was capable of being aminoacylated to at least 1400 pmol/A_{260} with FRS isolated from either *E. coli* or *Thermus thermophilus* (kindly provided by Dr. O. Lavrik, Institute of Bioorganic Chemistry, Novosibirsk, Russia). This confirms that the purified transcripts terminated with the appropriate CCA sequence. Initial rates from at least five tRNA concentrations were plotted using Eadie–

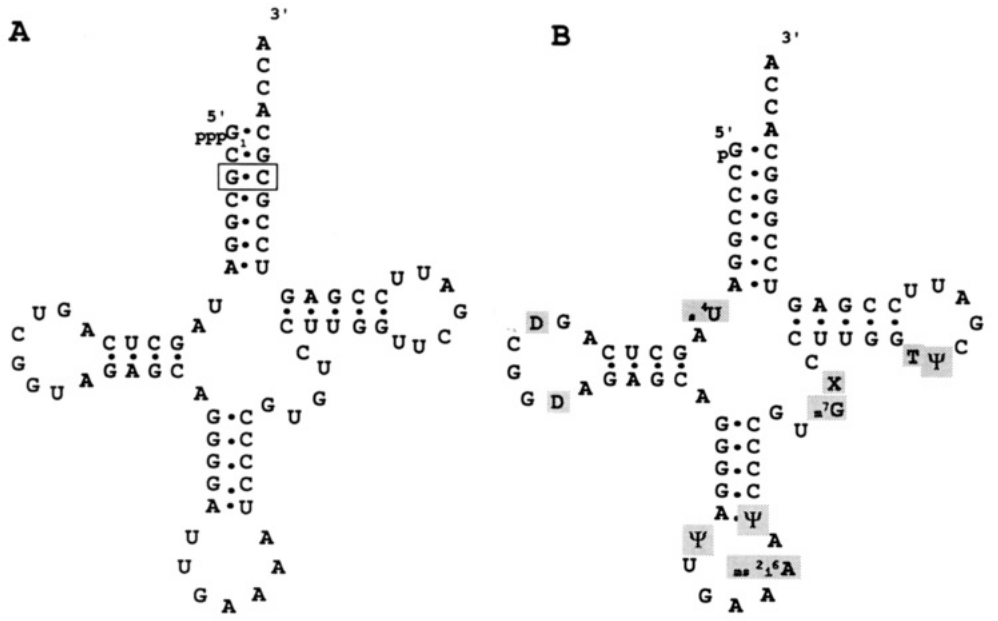

FIGURE 1: Comparison of the *E. coli* $tRNA^{Phe}$ transcript (A) and its natural counterpart (B). The boxed base pair in the transcript differs from that of the natural *E. coli* $tRNA^{Phe}$, and has been included in the construction of the *E. coli* $tRNA^{Phe}$ variants. The shaded regions in (B) indicate the 10 modified nucleotides present only in the natural tRNA.

Hofstee analysis and the K_M and V_{max} determined. The reported kinetic parameters represent the average of at least two separate determinations with correlation coefficients of at least 0.95, and the values of k_{cat}/K_M can be considered to be within 10% of the indicated values. The k_{cat}/K_M for the G34A transcript was measured under second-order conditions with substrate concentrations ranging from 0.2 to 2 μM tRNA. Under these conditions, the plot of the initial velocity vs the substrate concentration generated a line with a slope of V_{max}/K_M. Where very little aminoacylation occurred with up to 3 units/mL FRS and a line could not accurately be determined, the k_{cat}/K_M relative to the wild-type *E. coli* $tRNA^{Phe}$ transcript was estimated to be <0.001.

RESULTS

Aminoacylation of the Unmodified E. coli $tRNA^{Phe}$ Transcript. There are several reasons to expect that both *E. coli* $tRNA^{Phe}$ and the in vitro transcript used as a basis for comparison in this work (Figure 1) have tertiary structures very similar to that determined for yeast $tRNA^{Phe}$ by X-ray diffraction (Kim, 1978). First, the *E. coli* $tRNA^{Phe}$ sequences have the same number of nucleotides as yeast $tRNA^{Phe}$, with residues at the same positions forming base pairs. Second, *E. coli* $tRNA^{Phe}$ shares seven of nine tertiary interactions with yeast $tRNA^{Phe}$, and the two tertiary interactions that differ (G10-C25-U45, A26-G44) are common phylogenetic variants believed to have very similar structures (Sampson et al., 1990). Third, a single mutation in a single-stranded residue of the *E. coli* $tRNA^{Phe}$ transcript (U20G) makes it an excellent substrate for yeast FRS (Sampson & Uhlenbeck, 1988), an enzyme known to be sensitive to tRNA tertiary structure (Sampson et al., 1990). Finally, another mutation of a single-stranded residue (U60C) results in the *E. coli* $tRNA^{Phe}$ transcript being susceptible to specific cleavage with lead at a rate comparable to that of the yeast $tRNA^{Phe}$ transcript (Behlen et al., 1990). Since lead cleavage is also very sensitive to the precise folding of tRNA, the two tRNAs must have very similar structures.

The transcript in Figure 1, when properly renatured (Materials and Methods), is capable of being fully aminoacylated (1600 pmol/A_{260}) with *E. coli* FRS under a variety of buffer conditions. The K_M for the fully modified $tRNA^{Phe}$ is quite similar to that determined previously under slightly different conditions (Stulberg, 1967; Santi et al., 1971). In a buffer containing 15 mM $MgCl_2$, the transcript shows slightly higher K_m and k_{cat} values than the natural $tRNA^{Phe}$, resulting in a relative catalytic efficiency of 0.79 (Table I). As was found in the yeast $tRNA^{Phe}$ system, buffers containing lower concentrations of $MgCl_2$ resulted in reduced rates of ami-

Table I: Catalytic Parameters for Aminoacylation with *E. coli* FRS and Lead Cleavage Rates for *E. coli* $tRNA^{Phe}$ Mutants

mutation	K_M (nM)	k_{cat} (min^{-1})	relative k_{cat}/K_M	Pb cleavage (relative k_{obs})[a]
E. coli $tRNA^{Phe}$ transcripts	80	85	1.2	*b*
G3C70[c]	220	190	(1.0)	*b*
C3G70[c]	100	120	1.4	*b*
U60C	190	150	0.91	(1.0)
U59C	950	130	0.16	*b, d*
U16C	220	150	0.79	*b*
C17U	190	150	0.91	*b*
U20G	1000	210	0.24	0.15[a]
U20C	880	200	0.26	0.19
U20A	900	180	0.23	0.19
G34A			0.0025	0.89
G34C,A35U			<0.001	0.89
modified amber suppressor			<0.001	*b*
A36C	2500	35	0.016	*b*
G44C	3900	150	0.044	0.14
A26G,G44A	2400	150	0.072	0.88
U45G	1800	220	0.14	1.0
G10A,C25U	1500	90	0.069	0.53
G27A,C43U	330	185	0.65	1.0
G28A,C42U				
A73C	400	160	0.46	*b*
A73U	350	130	0.43	*b*
A73G	260	140	0.62	*b*

[a] Relative to U60C transcript, $k_{obs} = 5.3 \times 10^{-4}$ s^{-1}. All mutants for which lead cleavage rates are given contain U60C. In several cases, it was confirmed that including U60C had no additional effect over the first mutation in the aminoacylation reaction. [b] Values not determined. [c] When transcribed in the presence of GMP to generate a monophosporylated 5′ end, k_{cat}/K_M is comparable to the value obtained for the triphosphorylated substrate. [d] Mutation of this nucleotide expected to disrupt lead binding (Behlen et al., 1990). [e] Additional cleavage sites observed.

noacylation for the transcript. This is believed to be due to the lower stability of tRNA transcripts and possibly a weaker affinity for magnesium (Sampson & Uhlenbeck, 1988).

The in vitro *E. coli* tRNA^{Phe} transcript differs from the native *E. coli* tRNA^{Phe} in three significant ways. It lacks the 10 modifications present in the native tRNA, it contains a G3-C70 base pair instead of a C3-G70 base pair in order to improve transcription efficiency, and it has a 5′-terminal triphosphate instead of a monophosphate. The small differences in k_{cat} and K_M between the transcript and native tRNA^{Phe} under the conditions described are most likely due to the existence of modifications in the natural substrate. Several examples have been reported where transcripts have a higher K_M than their natural counterparts (Sampson & Uhlenbeck, 1988; Schulman & Pelka, 1988; Himeno et al., 1990). The presence of a 5′-triphosphate and a change in the sequence at positions 3–70 change the kinetic parameters only slightly (Table I). For this reason, all subsequent transcripts were generated with a 5′-triphosphate and the G3-C70 base pair to improve the transcriptional yield.

Identification of Recognition Nucleotides in E. coli tRNA^{Phe} Transcripts. As shown in Table I, U60C has a k_{cat}/K_M virtually identical to the wild type, suggesting that this residue is not a recognition nucleotide. This result permits the use of lead cleavage as an assay for the structural integrity of *E. coli* tRNA^{Phe} variants. It was shown by the use of yeast tRNA^{Phe} variants that the rate of lead-catalyzed cleavage is sensitive to structural changes induced by mutations in regions important for tRNA folding (Behlen et al., 1990). Because a U60C substitution confers lead-reactivity to the *E. coli* tRNA^{Phe} transcript, this structural probe can be used for variants of *E. coli* tRNA^{Phe} also. Therefore, by the application of this assay for structural integrity, mutants which disrupt aminoacylation can be categorized as those which misfold and those which fold normally and thus are impaired as a result of the loss of a recognition contact.

The nucleotides in the "variable pocket", 16, 17, 20, 59, and 60, described by Ladner et al. (1975) have been suggested to be important for protein–tRNA interactions. While nucleotide 60 in the T-loop was not found to be important in recognition, a U59C mutation results in a 6.3-fold decrease in the catalytic efficiency (Table I). Although the U59C mutant is presumably unable to coordinate lead for cleavage (Behlen et al., 1990), the crystal structure of this same region in yeast tRNA^{Phe} suggests that the conservative pyrimidine to pyrimidine change is unlikely to significantly affect folding. The single-stranded D-loop nucleotides 16 and 17 do not seem to interact with *E. coli* FRS since the conservative mutations U16C and C17U are virtually normal in aminoacylation kinetics. While the three mutants at position 20 have a 4–5-fold reduction in catalytic efficiency, they are found to cleave with lead at a reduced rate indicative of folding defects (Table I), and the U20G mutant shows several additional cleavage sites. Due to the misfolding of the position 20 mutants, it is difficult, in the absence of additional data, to determine whether any of the reduction in catalytic efficiency is due to the loss of a recognition contact.

As found for many other synthetases (Schulman, 1991), the maintenance of the wild-type anticodon nucleotides is critical for wild-type aminoacylation kinetics. The G34A mutation alone is enough to cause a 400-fold reduction in k_{cat}/K_M, and the A36C mutation results in a 60-fold reduction of k_{cat}/K_M (Table I). The amber suppressor transcript with both mutations G34C and A35U is reduced in catalytic efficiency by at least 1000-fold. Similarly, the activity of the modified amber suppressor tRNA isolated from an overproducing plasmid is reduced by at least 3 orders of magnitude. While the lead cleavage rate for the tRNA containing a mutation at nucleotide 36 was not determined, the mutations at 34 and 35 were shown to have little effect on the structure of *E. coli* tRNA^{Phe} in the vicinity of the lead binding site. The surprisingly poor aminoacylation of the amber suppressor tRNAs, as well as the other anticodon mutants, appears to be a direct consequence of the anticodon substitutions since all the tRNAs can be fully aminoacylated by FRS isolated from *T. thermophilus* (data not shown), indicating that they terminate correctly with a 3′-CCA.

Nucleotide changes at positions 26, 44, 45, 10, and 25 of *E. coli* tRNA^{Phe} all have an effect on aminoacylation, suggesting the participation of the central core of the tRNA in recognition by the synthetase. A26 and G44 are believed to form a "propellor twist" tertiary interaction very similar to the G26–A44 interaction seen in yeast tRNA^{Phe} (Quigley & Rich, 1976; Sampson et al., 1990). Although a mutation of G44C results in a reduction of the aminoacylation efficiency of the mutant by more than 20-fold, the low lead cleavage rate suggests that the mutant is not structurally similar to the wild type. The more structurally conservative double mutation A26G,G44A has proper structure based on lead cleavage kinetics, but exhibits a reduction in k_{cat}/K_M of 14-fold. This suggests that the propellor twist is involved in recognition. While G45, located above the 26–44 interaction in the molecule, is involved in a tertiary interaction with the base pair G10-C25 in the crystal structure of yeast tRNA^{Phe} (Quigley & Rich, 1976), an analogous hydrogen-bonding pattern with U45 of *E. coli* tRNA^{Phe} is expected, but not obvious (Sampson et al., 1990). When a G is substituted for U45 in the *E. coli* tRNA^{Phe} transcript, the catalytic efficiency is reduced 7-fold, but lead cleavage kinetics suggest no structural alteration. Similarly, a replacement of G10-C25 by A10-U25 reduces the k_{cat}/K_M by 14-fold, while lead cleavage indicates relatively little structural perturbation. These two experiments suggest that one or more residues in this tertiary interaction are involved in recognition as well. This region of recognition does not seem to extend below the A26-G44 pair since mutations at the base pairs 27-43 and 28-42 of the anticodon stem only reduce k_{cat}/K_M 1.5-fold relative to wild type. The role of the nucleotides in the base pair 11-24, located above the 10–25–45 interaction, was not evaluated.

The FRS from *E. coli* only uses the "discriminator" nucleotide at position 73 (Crothers et al., 1972) to a limited extent to identify its cognate tRNA. The A73C and A73U mutations reduce k_{cat}/K_M only 2.2-fold and 2.3-fold, respectively, while the more conservative A73G has an effect of less than 2-fold. These results indicate that nucleotide 73 participates in recognition only to a modest degree.

Recognition Information Obtained by Using Yeast tRNA^{Phe} Variants. The numerous available yeast tRNA^{Phe} mutants (Sampson & Uhlenbeck, 1988; Sampson et al., 1989, 1990, 1992) could potentially be used to confirm and supplement the recognition set identified by *E. coli* tRNA^{Phe} mutants. Despite 26 nucleotide differences between yeast and *E. coli* tRNA^{Phe} (Figure 2), the catalytic efficiency of aminoacylation of the yeast tRNA^{Phe} transcript by *E. coli* FRS is only reduced 9-fold (Table II). If the contribution of each recognition contact to k_{cat}/K_M is independent and remains constant across a variety of tRNA sequences (Sampson et al., 1992), one should be able to identify potential *E. coli* FRS recognition nucleotides by further reduction in the k_{cat}/K_M of yeast tRNA^{Phe} when those positions are mutated. In addition, it

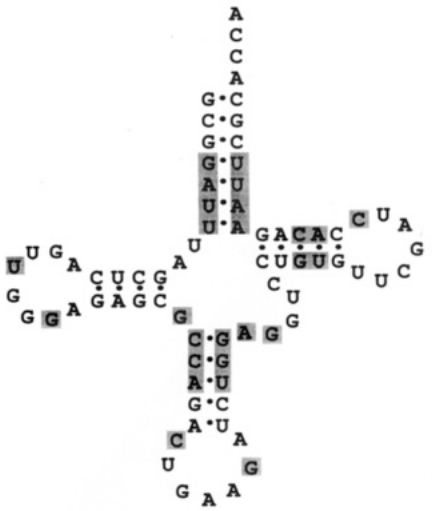

FIGURE 2: Secondary structure of the yeast tRNA^{Phe} transcript. The nucleotides that differ between the yeast and *E. coli* tRNA^{Phe} transcripts are shaded.

Table II: Catalytic Parameters for Aminoacylation with *E. coli* FRS and Lead Cleavage Rates for Yeast tRNA^{Phe} Transcripts

mutation	K_M (nM)	k_{cat} (min^{-1})	relative k_{cat}/K_M	Pb cleavage (relative k_{obs})
E. coli tRNA^{Phe}	220	190	8.9	
yeast tRNA^{Phe}	880	85	(1.0)	(1.0)
G34A			<0.01	0.93[a]
A35U			<0.01	0.86[a]
A36C			<0.01	*b*
C60U	560	65	1.2	0.08[c,d]
U59C	970	50	0.53	0.10[c,d]
A73U	300	5	0.17	0.47[a]
G20U	650	110	1.8	0.92[c]
G20A	550	70	1.3	0.85
A44U	900	20	0.23	0.67
G26A,A44U	250	15	0.62	0.62
G26A,A44G	900	85	0.98	0.54
G45U	780	80	1.1	0.84[e]
G20U,G26A, A44G,G45U	160	45	2.9	0.50
A9G,U12C,A23G	1400	40	0.30	0.71[c]
C13U,G22A,G46A	1500	50	0.35	0.44
G15A,C48U	910	50	0.57	1.2[c]
A38C	620	35	0.58	0.52[a]
G1A,C72U	1000	55	0.57	*b*

[a] Data taken from Sampson et al. (1992). [b] Values not determined. [c] Data taken from Behlen et al. (1990). [d] Low value attributed to disruption of the lead binding site (Behlen et al., 1990). [e] Behlen, personal communication.

might be possible to improve the k_{cat}/K_M of yeast tRNA^{Phe} as much as 9-fold by mutating 1 or more of the 26 nucleotides which differ.

Yeast tRNA^{Phe} mutants with changes at positions 34, 35, 36, 59, 60, and 73 were able to qualitatively confirm the conclusions made with *E. coli* mutants (Table II). Thus, all three anticodon mutations reduce aminoacylation significantly, the residue at position 60 has little effect, U59 is preferred over C59, and A73 is preferred over U73. However, some quantitative differences are seen when the same mutation is compared in the two tRNA^{Phe} backgrounds. While the anticodon mutations aminoacylate too poorly in either background to compare, a pattern is not evident when the effect of the other mutations in the two tRNAs is compared. For example, while U59C reduces k_{cat}/K_M more in *E. coli* tRNA^{Phe} than in yeast tRNA^{Phe} (6-fold and 2-fold, respectively), A73U has a larger effect on k_{cat}/K_M in yeast tRNA^{Phe} (6-fold) than in *E. coli* tRNA^{Phe} (2-fold). Thus, it is clear that despite their similar structures, some aspect of the interaction between the synthetase and its cognate tRNA differs from that with the heterologous tRNA although the regions that interact appear to be the same.

Mutations at position 20 in yeast tRNA^{Phe} were instrumental in resolving the ambiguity seen with mutations at the same position in *E. coli* tRNA^{Phe}. Unlike in *E. coli* tRNA^{Phe}, changing the residue at position 20 in yeast tRNA^{Phe} has no effect on the rate of lead cleavage (Behlen et al., 1990), so the importance of this position in recognition can be evaluated directly. If, in fact, U20 is recognized by *E. coli* FRS, it is expected that changing G20 in yeast tRNA^{Phe} to a U residue should improve the aminoacylation by FRS. As shown in Table II, the G20U mutation does indeed improve the k_{cat}/K_M of yeast tRNA^{Phe} while G20A has less of an effect. This indicates that position 20 is a recognition nucleotide for *E. coli* FRS, but contributes relatively little.

The nucleotide 44 which is involved in the propellor twist of *E. coli* tRNA^{Phe} was identified as a recognition element in the yeast tRNA^{Phe} background (Table II). Substitution of A44U reduces the k_{cat}/K_M by as much as 4-fold. However, one would expect that changing the three residues in yeast tRNA^{Phe} which differ (G26, A44, and G45) to the corresponding *E. coli* tRNA^{Phe} residues (A26, G44, and U45) should improve k_{cat}/K_M. Surprisingly, neither the single mutant G45U nor the double mutant G26A,A44G improved k_{cat}/K_M, although the double mutant showed a reduced rate of cleavage with lead and thus may not be correctly folded. Since it is possible that the two tertiary interactions act synergistically instead of independently, a quadruple mutant of yeast tRNA^{Phe} was prepared (G20U, G26A, A44G, and G45U) that introduced all the *E. coli* recognition nucleotides thought to be missing. While this quadruple mutant aminoacylates better than the G20U alone, thereby confirming the recognition role of nucleotides 26, 44, and 45, the lower overall rates of both aminoacylation and lead cleavage suggest some remaining defect in the folding of this mutant.

Additional yeast tRNA^{Phe} transcripts with mutations at positions untested with *E. coli* mutants were assayed with *E. coli* FRS. Mutations at nucleotides involved in triple interactions at 9–12–23 an 13–22–46 were found to have modest 3-fold effects on aminoacylation with *E. coli* FRS. However, a roughly corresponding reduction in the rate of lead cleavage was also observed, lending little information to the role of these nucleotides in recognition. While the lead cleavage rate indicates no structural effect of a mutation at the 15–48 interaction, a 1.7-fold reduction in k_{cat}/K_M is seen relative to the wild-type yeast tRNA^{Phe} transcript (Table II). Approximately the same effect on k_{cat}/K_M is observed for two additional mutants, A38C in the anticodon loop and G1A-C72U at the top base pair of the acceptor stem. While a mutation at 1–72 is not expected to affect the structure (the lead cleavage rate was not measured), the mutation in the anticodon loop causes a reduction in the cleavage rate of approximately 2-fold. With the yeast enzyme, the relative k_{cat}/K_M for the 9–12–23 mutant was reported to be reduced by no more than 1.2-fold, and the 13–22–46 interaction actually slightly increased the k_{cat}/K_M over the wild-type sequence in spite of the reduced lead cleavage rate (Sampson et al., 1990), suggesting that the structure of these mutants is not altered enough to reduce their ability to interact efficiently with yeast FRS. Additionally, it has been shown that the same substitution of a recognition nucleotide (U59C) has a much smaller impact on aminoacylation with *E. coli* FRS in the yeast tRNA^{Phe} background than in the homologous

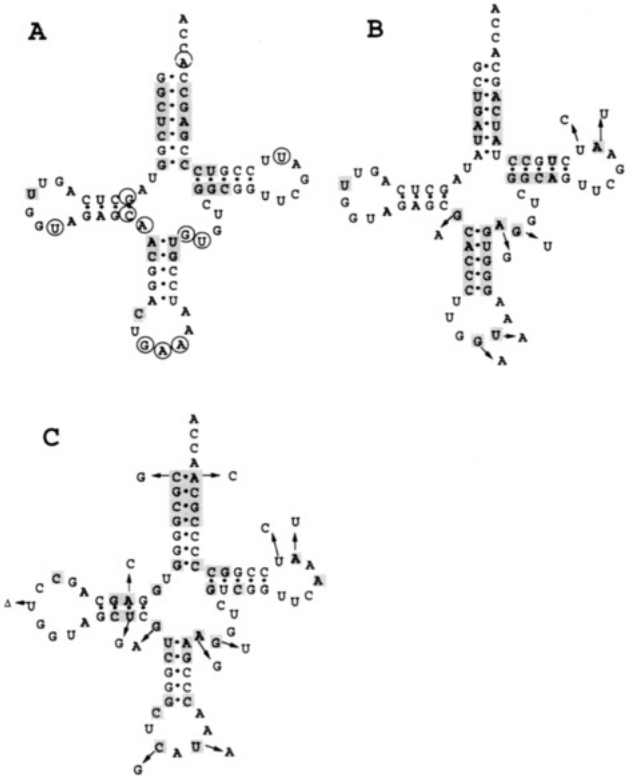

FIGURE 3: tRNA transcripts that test the model for *E. coli* tRNA^{Phe} recognition. The nucleotides that are shaded differ from *E. coli* tRNA^{Phe}. (A) *B. subtilis* tRNA^{Phe}. The 11 nucleotides proposed as recognition nucleotides are circled. (B) *E. coli* tRNA$^{Thr\ to\ Phe}$. (C) *E. coli* tRNA$^{fMet\ to\ Phe}$. For (B) and (C), the nucleotide substitutions are indicated by arrows. (Δ) represents the deletion of the indicated nucleotide.

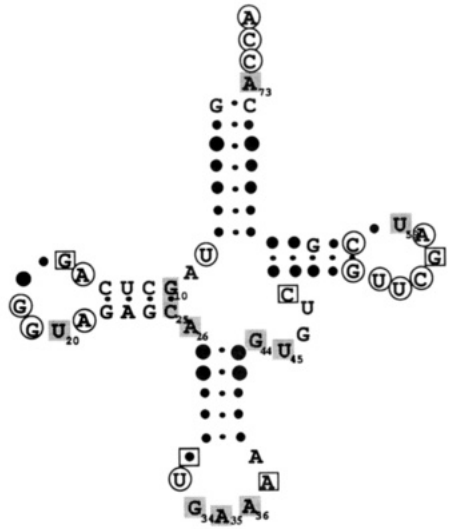

FIGURE 4: *E. coli* tRNA^{Phe} recognition set. The recognition nucleotides for *E. coli* FRS are shaded. The substitutions that have been found to have little effect on aminoacylation kinetics are represented by a dot. The dot size is proportional to the number of tRNA variants tested that support the elimination of that position as a recognition nucleotide. The nucleotides conserved in nonmitochondrial elongator tRNAs are circled, and those semiconserved are boxed.

Table III: Catalytic Parameters for Aminoacylation with *E. coli* FRS and Lead Cleavage Rates for Transcripts Testing the Recognition Model

mutation	K_M (nM)	k_{cat} (min^{-1})	relative k_{cat}/K_M	Pb cleavage (relative k_{obs})
E. coli tRNA^{Phe}	220	190	(1.0)	(1.0)[a]
B. subtilis tRNA^{Phe}	200	190	1.1	*b*
tRNA^{Thr}			<0.001	*b*
tRNA$^{Thr\ to\ Phe}$	360	190	0.61	1.0
tRNA$^{Met\ to\ Phe}$	1900	31	0.019	0.10

[a] Lead cleavage of U60C mutant. [b] Values not determined.

background. Taken together, this information suggests that the participation of some of these nucleotides in recognition cannot be ruled out. Given that the relationship of the mutations to the aminoacylation kinetics in different backgrounds is not a simple one, caution should be taken when interpreting the effects of nucleotide changes within sequences other than the cognate tRNA.

Testing the Model for Recognition. A model can be proposed for the recognition nucleotides of *E. coli* tRNA^{Phe} by *E. coli* FRS (Figure 4). The recognition set appears to consist of 11 nucleotides: U20 in the D-loop, U59 in the T-loop, A26, G44, G10, C25, and U45 in the central core, A73 and the three anticodon nucleotides G34, A35, and A36.

tRNA^{Phe} from *Bacillus subtilis* (Figure 3A) would be expected to be a good substrate for *E. coli* FRS. Even though the sequence differs from that of *E. coli* tRNA^{Phe} at 21 positions, it has the same 11 recognition residues. In support of the model, the *B. subtilis* transcript was shown to exhibit catalytic parameters practically identical to that of the cognate substrate for *E. coli* FRS (Table III). The nucleotides that differ between the two tRNAs therefore can be eliminated as possible recognition nucleotides.

The best test of the validity of a proposed recognition set is to substitute the proposed recognition nucleotides into a tRNA that is a poor substrate for the enzyme and demonstrate that efficient aminoacylation occurs (Schulman & Pelka, 1988; Sampson et al., 1989). Such a "recognition swap" experiment was attempted with *E. coli* tRNA^{Thr} whose sequence differs from tRNA^{Phe} at 32 positions (Figure 3B). Wild-type tRNA^{Thr} transcript (from a plasmid kindly supplied by Dr. L. Schulman, Albert Einstein College of Medicine, New York) aminoacylates with a relative k_{cat}/K_M at least 3 orders of magnitude below that for tRNA^{Phe} (Table III). The model for recognition by *E. coli* FRS suggests that six nucleotides changes, G26A, G35A, U36A, A44G, G45U, and A59U, would be necessary to change tRNA^{Thr} into a substrate for FRS. These six substitutions were made in *E. coli* tRNA$^{Thr\ to\ Phe}$, along with U60C, to provide an active lead binding site to assess the structure (Figure 3B). This transcript is an excellent substrate for *E. coli* FRS (Table III) in support of the model for recognition, exhibiting the same k_{cat} and a K_M increased only 1.5-fold relative to *E. coli* tRNA^{Phe}. This result also allows the elimination of the 26 nucleotides in tRNA$^{Thr\ to\ Phe}$ that differ from *E. coli* tRNA^{Phe} as possible recognition nucleotides.

Although a fully active substrate was obtained upon substitution of the recognition nucleotides into *E. coli* tRNA^{Thr}, a similar substitution of the recognition nucleotides into *E. coli* tRNA^{fMet} was only partially successful in conferring wild-type kinetics to a noncognate substrate. Previous experiments by others have shown that *E. coli* FRS does not aminoacylate tRNA^{fMet}. However, introduction of the phenylalanine anticodon nucleotides (C34G and U36A) improves aminoacylation to a low but detectable level (Chattapadhyay et al., 1990). On the basis of the model of *E. coli* FRS recognition, tRNA^{fMet} should be converted into a better substrate for *E. coli* FRS by changing four additional recognition nucleotides (G26A, A44G, G45U, and A59U) and deleting C17.1 which could potentially disrupt the D-loop structure (Davis et al., 1986; Giegé et al., 1990). In preparing tRNA$^{fMet\ to\ Phe}$, the

unusual $tRNA^{fMet}$ base pairs C1-A72 and A11-U24 were also changed to G1-C72 and C11-G24, and the lead cleavage site was introduced (U60C). As shown in Figure 3C, $tRNA^{fMet\ to\ Phe}$ aminoacylates with a k_{cat}/K_M 50-fold below *E. coli* $tRNA^{Phe}$. While no aminoacylation kinetics are available for $tRNA^{fMet}$ with a phenylalanine anticodon, $tRNA^{fMet\ to\ Phe}$ appears to be a significantly better substrate for *E. coli* FRS. The most likely reason that the $tRNA^{fMet\ to\ Phe}$ transcript does not aminoacylate to the wild-type level is that it does not fold properly as judged by the low rate of lead cleavage. However, negative elements may exist in *E. coli* $tRNA^{fMet}$ that preclude efficient aminoacylation with *E. coli* FRS. Indeed, the unusual base pairs in the anticodon stem of $tRNA^{fMet}$ have been correlated to initiator activity (Seong & RajBhandary, 1987), and may dictate a certain conformation incompatible with the efficient aminoacylation by *E. coli* FRS. Thus, though partially successful, this experiment may represent another example where substitution of the recognition nucleotides is not sufficient to obtain a fully active substrate.

DISCUSSION

The recognition set for *E. coli* FRS elucidated by this study includes 11 of the 76 nucleotides in *E. coli* $tRNA^{Phe}$ (Figure 4). These nucleotides are G10, U20, C25, A26, G34, A35, A36, G44, U45, U59, and A73, with substitutions at the anticodon nucleotides having the greatest impact on the aminoacylation rate. These 11 nucleotides comprise a relatively large recognition set as compared with other tRNAs (Schulman & Pelka, 1983; Hou & Schimmel, 1988; Sampson et al., 1989; Himeno et al. 1990) and imply an extensive region of contact between FRS and $tRNA^{Phe}$. It is, however, important to note that the existence of a recognition nucleotide does not necessarily imply a direct contact between the enzyme and the nucleotide ring. Instead, the recognition nucleotide may be needed to allow a proper interaction with the synthetase to occur in some other way. For example, residues U1 and A72 are formally recognition nucleotides in $tRNA^{Gln}$ (Jahn et al., 1991), but do not form sequence-specific contacts with the protein in the cocrystal structure (Rould et al., 1989).

There is considerable independent evidence that suggested that some of the nucleotides identified here are important in recognition for *E. coli* FRS. Two in vitro experiments by others have suggested that D-stem residues of *E. coli* $tRNA^{Phe}$ are contacted by *E. coli* FRS. First, methylation of G10 was found to reduce the V_{max} for aminoacylation by 10-fold (Roe et al., 1973). Second, nucleotide 24 was shown to be protected from alkylation in the presence of *E. coli* FRS (Ankilova et al., 1975). Although G24 was not specifically implicated in recognition by our study, the protection of this region suggests that the synthetase is indeed making contacts with nucleotides very close in three-dimensional structure to those implicated in recognition by in vitro aminoacylation. Finally, while not proven, the participation of nucleotide 44 in tRNA recognition might explain the isolation of five independent G44A mutants identified by their ability to deattenuate protein synthesis through the FRS control region in vivo (Vacher et al., 1985). Indeed, the conservation of nucleotides 26, 44, and 45 among prokaryotic $tRNA^{Phe}$s (Sprinzl et al., 1989) suggests that the specific sequence or possibly the inherent structure of these nucleotides has been preserved among prokaryotic species for recognition by the synthetase.

The identity set for *E. coli* $tRNA^{Phe}$ determined by in vivo suppressor analysis has been reported (McClain & Foss, 1988a) and can be compared to the recognition set described here. Where possible, the same substitutions that were suggested by the suppressor study to have an influence on identity were tested with transcripts in vitro. The identity set consists of 10 nucleotides: 20, 27, 28, 42, 43, 44, 45, 59, 60, and 73, 5 of which appear to be both recognition and identity nucleotides. The recognition nucleotides in agreement with the identity set are 20, 44, 45, 59, and 73. Substitution of the nucleotides at 60 and the base pairs at 27-43 and 28-42 had only minor effects on aminoacylation in vitro. Thus, the maintenance of these nucleotides does not seem to be required for the productive interaction with FRS, but may be necessary to deter the noncognate synthetases in *E. coli* from interacting with $tRNA^{Phe}$. Alternatively, the reduced ability of these mutants to function in vivo may be attributed to a different step of translation.

The nucleotides 10, 25, 26, 34, 35, and 36 were found to be important for recognition but are not present among the identity nucleotides. Substitutions at nucleotides 10 and 25 in the D-stem will presumably influence the identity of $tRNA^{Phe}$ in vivo, but were not tested by substitutions into the amber suppressor $tRNA^{Phe}$. However, substitutions at the adjacent 11-24 base pair, and the 9–12–23 triple interaction, were made but could not be evaluated since the mutants were inactive (McClain & Foss, 1988a). Although 26 is considered to be a recognition nucleotide, the contributions of nucleotides 26 and 44 were not delineated in vitro since their simultaneous mutation was seen to be necessary for the maintenance of the proper structure of the *E. coli* $tRNA^{Phe}$ transcript.

The most notable difference between the recognition set and the identity set is the participation of the anticodon nucleotides. Because the identity set was determined using an amber suppressor tRNA, the effects of mutations of the anticodon nucleotides could not be examined. The *E. coli* $tRNA^{Phe}$ amber suppressor (CUA anticodon) is an efficient suppressor and is correctly aminoacylated by FRS as indicated by the presence of phenylalanine at the amber codon positions in suppressed protein (Normanly et al., 1986a; McClain & Foss, 1988a). However, the analogous mutant transcript was shown to aminoacylate with a catalytic efficiency at least 3 orders of magnitude below that seen for the wild-type tRNA. Similar results were obtained with the modified suppressor tRNA isolated from an *E. coli* strain which overproduces it. It is striking that an inefficient tRNA in vitro maintains its identity in vivo. Since many suppressor tRNAs are only effective when present on multicopy plasmids (Normanly et al, 1990), the intracellular tRNA concentration may be sufficient to overcome the elevated K_M. In addition, the suppressor may contain enough negative determinants to prevent even a competitive level of aminoacylation by other synthetases. Even though the identity set was obtained starting with a severely kinetically damaged tRNA, many of the same residues found with in vitro experiments were identified.

Although substitutions at 20 and 73 affect aminoacylation kinetics in vitro, they have a relatively minor influence with respect to other recognition nucleotide substitutions; their inclusion in the recognition set was primarily based on their effects in the yeast $tRNA^{Phe}$ background. The presence of 20 and 73 among the identity nucleotides reiterates their importance in tRNA discrimination. It was demonstrated in vivo that a U20G substitution into a *Halobacterium volcanii* $tRNA^{Phe}$ amber suppressor results in the substitution of several other amino acids into suppressed protein (McClain & Foss, 1988a). The *E. coli* $tRNA^{Phe}$ amber suppressor will be aminoacylated with glycyl-tRNA synthetase if A73 is mutated to U (McClain et al., 1990) and with glutaminyl-tRNA

synthetase if a A73G substitution is made (McClain & Foss, 1988a). While there are several explanations for such an observation, it is possible that nucleotides 20 and 73 are more important in preventing noncognate interactions from occurring than in providing contacts for the correct synthetase. It is important to mention that U20 is normally modified to D in the natural substrate. Because the unmodified transcript is nearly as active, it is not likely that the modification itself is required for recognition (Muramatsu et al., 1988), but may be necessary in vivo for discrimination.

The recognition set described herein can easily serve to discriminate between tRNA^{Phe} and all other tRNAs in *E. coli* [see Sampson et al. (1992)]. In comparing all *E. coli* tRNA sequences, we noted that no other sequence with the same secondary structure as tRNA^{Phe} contains more than 7 of the 11 recognition nucleotides (Komine et al., 1990). The two tRNA sequences that include seven recognition nucleotides, tRNA^{Val}(UAC) and tRNA^{Trp}(CCA), both have two anticodon nucleotides that differ from tRNA^{Phe}, presumably sufficient to preclude interaction with *E. coli* FRS. Those tRNAs in *E. coli* that share two anticodon nucleotides with tRNA^{Phe} and might be expected to aminoacylate with phenylalanine have a different number of nucleotides in the D-loop and/or variable loop. This suggests that the recognition set in combination with structural variety among tRNAs is sufficient for selective aminoacylation of tRNA^{Phe} by its cognate synthetase. Indeed, *E. coli* tRNA^{Thr}, which has 5 of the 11 recognition nucleotides and is not a substrate for *E. coli* FRS, can be substituted with the remaining 6 nucleotides suggested by the model and is subsequently efficiently aminoacylated by *E. coli* FRS.

The recognition nucleotides can be considered to reside in one of four general regions of the tRNA tertiary structure: the variable pocket (20 and 59), central core (10, 25, 26, 44, and 45), anticodon loop (34, 35, and 36), and acceptor end (73). All four regions are familiar areas for other tRNA synthetase interactions both in vivo and in vitro [see Schulman (1991) and references cited therein], although the quantitative contribution of each area to the overall catalytic efficiency of aminoacylation in vitro can be quite different. While substitution of the anticodon nucleotides in *E. coli* tRNA^{Phe} has at least a 60-fold effect on k_{cat}/K_M, changing the recognition nucleotides in the anticodon of other tRNAs is known to reduce the catalytic efficiency by anywhere from several orders of magnitude in *E. coli* tRNA^{fMet} (Schulman & Pelka, 1983) to only 4–5-fold in yeast tRNA^{Tyr} (Bare & Uhlenbeck, 1986). Substitution of nucleotide 73 has also been shown to give rise to a wide range of effects on catalytic efficiency with different tRNA synthetase systems. Although the impact of a substitution at this position in *E. coli* tRNA^{Phe} is only slightly more than 2-fold, effects of more than 3 orders of magnitude have been seen with *E. coli* tRNA^{Asp} (Hasegawa et al., 1989). Though some of these differences may be a result of different buffer and salt conditions, it seems that the different synthetases are quite idiosyncratic in the way they contact their substrates even if they rely on the same regions of the tRNA for recognition.

The structural consequences of substitutions in *E. coli* tRNA^{Phe} have proven to be unpredictable, in spite of the fact that the tertiary structure is thought to resemble that of yeast tRNA^{Phe}. Although the crystal structure helped to predict isomorphic nucleotide interactions in yeast tRNA^{Phe} and thereby permitted the predominantly successful design of mutant tRNAs with unaltered structures (Sampson et al., 1990), unanticipated folding anomalies have been much more prevalent for mutants of *E. coli* tRNA^{Phe}. The most surprising example of this is the substitution of the nucleotide at position 20 in *E. coli* tRNA^{Phe}. Because position 20 is not believed to interact with other nucleotides in the tertiary structure, its substitution was not predicted to alter the folding. All three nucleotide replacements affected the lead cleavage kinetics of the resultant transcript, with a G replacement not only reducing the rate of lead cleavage but also introducing additional cleavage targets. Because this mutation introduces three consecutive G's in the D-loop of a G-C-rich molecule, alternate conformations may be made more favorable. However, the deterimental effect of the other two substitutions, U20 to A or C, is not understood. It is possible that the function of this nucleotide, which is modified to a D in the natural substrate, is to stabilize the structure in this region. It is known that unmodified transcript tRNAs are less stable and their conformations are considered to be more flexible than for their modified counterparts (Sampson & Uhlenbeck, 1988; Perret et al., 1990). Some modifications are specifically thought to be required for tRNA stability in thermophiles (Davanloo et al., 1979). The *E. coli* tRNA^{Phe} transcript requires renaturation after storage at −20 °C, supporting the idea that alternate conformations are more prevalent in the unmodified than the modified form.

The notion that mutations in the D-loop may change the structure of modified tRNA^{Phe} has, however, been suggested by experiments in vivo. McClain and Foss (1988c) showed that the addition of a nucleotide in the β region of the D-loop prevented the tRNA from being normally processed, implying a structural defect in the precursor tRNA that made the substrate unsuitable for RNase P. This effect was compensated for by the coincident substitution of U59A, suggesting the sequences of the T-loop close to the D-loop nucleotides influence the structure. For this reason, it cannot be ruled out that the N20,C60 double mutant transcript required for structure evaluation by lead cleavage is exhibiting behavior that is a result of the proximity of the two mutated bases in the three-dimensional structure, and not a result of the 20 mutation by itself.

The substitution of nucleotides involved in the propellor twist was also found to have an impact on the folding of *E. coli* tRNA^{Phe}. The single substitution of G44C reduced the lead cleavage rate by 7-fold, suggesting that the resultant interaction at the propellor twist, A26–C44, was not isomorphic to the wild-type A26–G44 interaction. Although a putative propellor twist pair A26–C44 can be shown to exist naturally in the tRNA^{Arg,Cys,Gln,Leu,Ser,Tyr} of *E. coli* (Komine et al., 1990), the proposed secondary structures of these tRNAs are not considered to be equivalent to that of tRNA^{Phe} and therefore may represent slightly different folded structures. Because the G44C mutant was constructed on the basis of a similar mutant that suggested the requirement of G44 by suppressor analysis, the misfolding of this mutant is notable. Both propellor twist mutants constructed in our study, G44C and the double mutant A26G,G44A, significantly reduced k_{cat}/K_M. However, only the latter supported a normal lead cleavage rate, implying that the reduced k_{cat}/K_M resulted because of the loss of a recognition contact. This demonstrates the critical need for evaluating the structural character of tRNA mutants before a valid conclusion can be made regarding their effects on aminoacylation.

The results obtained from yeast tRNA^{Phe} mutants with *E. coli* FRS indicate that caution must be used in interpreting mutation data in backgrounds other than the cognate background. Several examples demonstrate that the substitutions

in yeast $tRNA^{Phe}$ do not always quantitatively (and sometimes qualitatively) reflect the activity of the analogously substituted *E. coli* $tRNA^{Phe}$. The inability to predict the effects of point mutations in yeast $tRNA^{Phe}$ with data derived from *E. coli* $tRNA^{Phe}$ mutants suggests that "recognition swaps" and perhaps also "identity swaps" will not always be easily obtained. Although the *E. coli* $tRNA^{Thr\ to\ Phe}$ experiment proved successful, the *E. coli* $tRNA^{fMet\ to\ Phe}$ construct was not fully active with *E. coli* FRS, and the significant improvement of yeast $tRNA^{Phe}$ as an *E. coli* FRS substrate was not seen without the coincident substitution of all four recognition nucleotides absent in yeast $tRNA^{Phe}$. Although it cannot be ruled out that the failure to convert the $tRNA^{fMet\ to\ Phe}$ substrate is a result of the presence of negative determinants within $tRNA^{fMet}$, the yeast $tRNA^{Phe}$ example seems to imply that the nucleotides may be subtly impacted by those around them. In fact, the nucleotides that reside close to one another in the tertiary structure may define a unique structural feature to that region of the tRNA. Therefore, the interaction of substrates having different combinations of nucleotides with FRS may have a different topology than that of the cognate substrate, resulting in mutations having different effects in the two backgrounds. Regardless of the explanation, it is clear that the additivity of the effects of mutations is not always perfect, and may depend on their context.

Because the recognition sets for phenylalanyl-tRNA synthetases from three different sources have been determined (Sampson et al., 1989; Nazarenko et al., 1992; results here), the concept of the conservation of the "second genetic code" (deDuve, 1988) can be addressed. The recognition of $tRNA^{Ala}$ by its cognate synthetase from both prokaryotes and eukaryotes was found to require primarily the G3-U70 base pair in the acceptor stem (Hou & Schimmel, 1989). Early results showed that although $tRNA^{Phe}$ from a prokaryotic source did not aminoacylate with FRS from a eukaryotic source and vice versa, *B. subtilis* FRS could aminoacylate *E. coli* $tRNA^{Phe}$ [Jacobson et al. (1971) and references cited therein]. These results suggested that although the recognition appeared to be conserved within prokaryotic species, it was not conserved across more evolutionarily diverged species. While the phenylalanyl synthetases from both yeast and *E. coli* have $\alpha_2\beta_2$ subunit structure, little homology has been found between them (Mirande, 1991; Sanni et al., 1988). Although the recognition sets for yeast and human FRS are quite similar, *E. coli* FRS relies on an additional region of its cognate tRNA to distinguish it from others that was shown to have little effect upon recognition by synthetases from eukaryotic species. All three synthetases recognize nucleotides at the end of the acceptor stem, in the variable pocket (although the nucleotide at 20 is different in human and yeast $tRNA^{Phe}$ than in *E. coli* $tRNA^{Phe}$), and in the anticodon, but the quantitative effects of mutations in these regions on the catalytic parameters differ between synthetases. Sampson et al. (1988) showed that the failure of yeast FRS to cross-aminoacylate *E. coli* $tRNA^{Phe}$ was a result of the nucleotide sequence at position 20, and the results here demonstrate that the identity of the nucleotides at 20, 26, 44, and 45 in yeast $tRNA^{Phe}$ prevents efficient aminoacylation by *E. coli* FRS. Thus, while some aspects of the recognition have been conserved, the mechanism by which the phenylalanyl-tRNA synthetases recognize their substrates seems to have diverged with the species.

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