Articles

Recognition of Yeast tRNA^{Phe} by Its Cognate Yeast Phenylalanyl-tRNA Synthetase: An Analysis of Specificity†

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ABSTRACT: A kinetic analysis of aminoacylation of mutant yeast tRNA^{Phe} transcripts by its cognate yeast phenylalanyl-tRNA synthetase (FRS) reveals five nucleotides in tRNA^{Phe} as major recognition sites for FRS. The aminoacylation kinetics for two double mutants suggest that each of the five recognition sites contributes independently to k_{cat}/K_M. Measured kinetic values for the mutants presented here and those reported previously were then used to calculate the predicted k_{cat}/K_M of misacylation for a number of noncognate tRNAs. The predicted k_{cat}/K_M values are consistent with values measured by other investigators and thus support the five-nucleotide recognition model. The k_{cat}/K_M of misacylation for all known yeast tRNAs has been calculated on the basis of this model, and the specificity of FRS for tRNA^{Phe} in yeast is discussed.

The specific aminoacylation of tRNAs by their cognate aminoacyl-tRNA synthetases is required to maintain the fidelity of protein synthesis. The identification of sequence-specific and structural elements in tRNAs that determine this specificity has been a major focus of study [reviewed in Schulman (1991)]. One effective way to determine these elements has been to introduce specific mutations in tRNAs and determine their effect on the in vitro aminoacylation kinetics with the cognate aminoacyl-tRNA synthetase. We have previously shown that a T7 RNA transcript corresponding to yeast tRNA^{Phe} is a good substrate for yeast phenylalanyl-tRNA synthetase (FRS) in buffers containing 15 mM $MgCl_2$ (Sampson & Uhlenbeck, 1988). This observation permits the use of mutant T7 tRNA transcripts to identify nucleotides in tRNA^{Phe} important for aminoacylation by FRS.

We have previously prepared a large number of mutant yeast tRNA^{Phe} transcripts and determined their steady-state kinetics of aminoacylation using purified yeast FRS. In an extensive analysis of the 21 nucleotides involved in the 9 tertiary interactions in tRNA^{Phe}, we found that mutations that disrupt a given tertiary interaction often reduce the k_{cat}/K_M of aminoacylation by FRS (Sampson et al., 1990). However, when the tertiary interaction was replaced by a compensatory change present in other tRNAs, the kinetics of aminoacylation by FRS were restored to wild-type values. This suggested that while the tertiary nucleotides are important for maintaining the proper folding of the tRNA, they do not appear to contribute sequence-specific contacts with the enzyme.

The five single-stranded nucleotides G20, G34, A35, A36, and A73 are located in three separate regions of tRNA tertiary structure and were previously identified as important for the recognition by FRS (Bruce & Uhlenbeck, 1982; Sampson & Uhlenbeck, 1988; Sampson et al., 1989). Introducing these five nucleotides into four other tRNA sequence backgrounds converted them into near-normal substrates for FRS (Sampson et al., 1989). These additional active substrates for FRS made it possible to eliminate a substantial number of nucleotides in tRNA^{Phe} as specific recognition elements for FRS, suggesting that only the five nucleotides are required to define tRNA^{Phe} as a specific substrate for FRS.

In this paper, we refine our model of FRS recognition by determining the aminoacylation kinetics for mutant tRNA^{Phe} transcripts having nucleotide substitutions at positions known to be important for FRS recognition and at other positions not previously tested directly. In addition, double mutants were constructed having nucleotide changes in two of the three known recognition regions to test whether these regions contribute independently to FRS recognition. The kinetic data for these mutants along with those previously reported are then used to calculate predicted k_{cat}/K_M values for several noncognate tRNAs and compare them to measured values determined by other investigators. This analysis is then applied to all known yeast tRNAs, providing a basis for examining the specificity of FRS for its cognate tRNA^{Phe}.

Materials and Methods

Deoxyoligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer (Foster City, CA). [^{3}H]Phenylalanine (60 Ci/mmol) was purchased from Amersham. T7 RNA polymerase was purified from *Escherichia coli* BL21 harboring the plasmid pAR1219 (Davanloo et al., 1984). Purified yeast phenylalanyl-tRNA synthetase (FRS) was a generous gift of P. Remy (Strasbourg).

Plasmids containing a mutant yeast tRNA^{Phe} gene flanked by the T7 RNA polymerase promoter and a *Bst*NI restriction site were constructed by methods previously described (Sampson & Uhlenbeck, 1988). In most cases, the tRNA genes containing point mutations were constructed by ligating a single mutagenic deoxyoligonucleotide with the five wild-type oligomers into pUS618 (a derivative of pUC18; C. Stover, unpublished results) and transforming the resulting mismatched DNA duplex. The mutants having anticodon and

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base-pair changes were constructed from two or four mutant oligomers. The position 73 mutants were constructed by subcloning 2 complementary 16-base oligonucleotides having a degeneracy at position 73 in the tRNA gene between the *Eco*RI and *Bam*HI restriction sites of the wild-type $tRNA^{Phe}$ plasmid (p67YF0). The double mutants involving changes at position 20, 34, and 73 were obtained by subcloning restriction fragments from the appropriate single mutants utilizing the unique internal *Bgl*II and *Eco*RI restriction sites. All plasmids were identified by sequencing mini-prep DNA and confirmed by sequencing $CsCl_2$-purified DNA.

Runoff transcription of *Bst*NI-linearized plasmid DNA by T7 RNA polymerase was performed as described previously (Sampson & Uhlenbeck, 1988). All transcripts were purified to single-nucleotide resolution by preparative polyacrylamide gel electrophoresis. The transcripts discussed in this paper were prepared with 5′-terminal triphosphates because previous experiments have shown that the extra two phosphates do not affect the aminoacylation kinetics. The yield of transcription for the mutant having an A at the first position was approximately 10-fold less than for the wild-type transcript. The 5′-terminal nucleotide for this mutant was confirmed to be the expected A (data not shown).

Steady-state aminoacylation kinetics for each mutant transcript were performed in a buffer containing 30 mM Hepes–KOH (pH 7.5), 15 mM $MgCl_2$, 25 mM KCl, 4.0 mM DTT, 2.0 mM ATP, and 10 μM [^{3}H]phenylalanine as described in Sampson and Uhlenbeck (1988). All mutant tRNA transcripts, with the exception of the double mutants, could be aminoacylated to a final level of 1200–1400 pmol/A_{260} unit at a concentration of FRS of 45 nM, confirming their structural and conformational purity. Kinetic determinations were performed using five concentrations of tRNA ranging from 0.10 to 5.0 μM depending on the mutant tRNA at a fixed FRS concentration of 1 nM. The k_{cat} and K_M values reported in Table II are the average of two separate determinations and can be considered to be ±10% of the indicated values.

Results and Discussion

Rationale. Because the primary focus of the experiments presented here is on the specific FRS recognition nucleotides, mutant tRNAs were designed to minimize any structural changes of the tRNA as defined by the crystal structure of the native yeast $tRNA^{Phe}$ (Kim et al., 1974a,b; Robertus et al., 1974; Ladner et al., 1975). To test for any structural effect as a result of a given mutation, the rate of lead-specific cleavage at U_{17} was determined. This cleavage reaction requires a specific binding site for Pb^{2+} (Brown et al., 1985) and has been shown to be a sensitive assay for the tertiary folding of the $tRNA^{Phe}$ transcript in the region of the molecule where the T-loop and D-loop interact (Behlen et al., 1990). The sensitivity of this reaction to structural changes as far away as the anticodon loop has also been reported (Krzyzosiak et al., 1988). The rate of lead cleavage for many of the mutants studied in this paper was reported previously (Behlen et al., 1990), and the remainder are given in Table I.

The mutant tRNAs examined here involve substitutions at positions G20, G34, A35, and A36, shown previously to be important for the recognition by FRS (Bruce & Uhlenbeck, 1982; Sampson et al., 1989), as well at positions not previously tested directly. These include U16 and U17 in the D-loop, U59 and C60 and the adjacent G53–C61 base pair in the T-stem-loop, A73 and the first base pair in the acceptor stem, and A38 and the last two base pairs of the anticodon stem (Figure 1). It is convenient to discuss the results for these mutations in three groups corresponding to the three regions of the tertiary structure in which they reside.

Table I: Rates of Lead Cleavage for Mutant $tRNA^{Phe}$ Transcripts

mutation	rel K_{obs}	ref
$tRNA^{Phe}$ transcript	(1.0)	
A73U	0.47	
A73C	0.83	
G20A	0.85	*a*
G20U	0.92	*a*
U59C	0.10	*a*
C60U	0.08	*a*
G34A	0.93	
U16C,U17C	1.33	*a*
A35U	0.86	
A38C	0.52	
G20A,A73U	0.93	
G34A,A73U	0.84	
G53C,C61G	0.08	*a*

[a] Behlen et al. (1990).

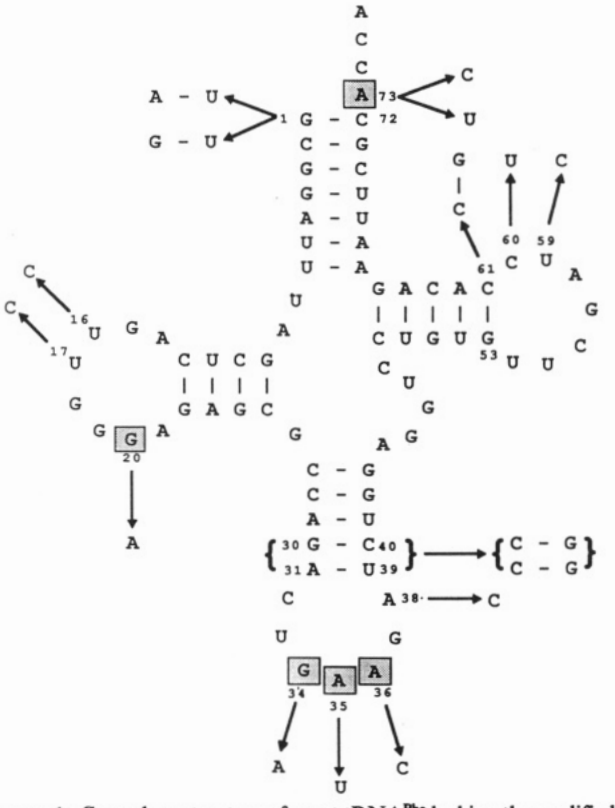

FIGURE 1: Secondary structure of yeast $tRNA^{Phe}$ lacking the modified nucleotides. The five FRS recognition sites are indicated by shading. Arrows indicate specific mutations discussed in this work.

Terminal Acceptor Stem Nucleotides. The 3′-terminal nucleotides C74, C75, and A76 are conserved in all tRNAs and therefore could not be discriminatory. However, the single-stranded nucleotide at position 73 varies among yeast tRNAs and was originally proposed to be discriminatory for all tRNAs on the basis of theoretical grounds (Crothers et al., 1972). The first experimental evidence that yeast FRS inspects A73 was suggested from misacylation experiments using a variety of *E. coli* tRNAs (Roe & Dudock, 1972; Roe et al., 1973). More recently, we have shown that introducing eight nucleotide changes into yeast $tRNA^{Arg}$ resulted in a tRNA that was an excellent substrate for FRS when it contained A73 whereas a mutant which retained G73 was a 5-fold worse substrate for FRS (Sampson et al., 1989).

To further test the importance of A73 for FRS recognition, we examined the aminoacylation kinetics of two mutant transcripts having either a C73 or a U73. The k_{cat}/K_M values for the A73C and A73U mutants are 8- and 12-fold lower,

Table II: Aminoacylation Kinetics of tRNA^{Phe} Mutants

mutation	K_M (μM)	$k_{cat(rel)}$	k_{cat}/K_M
wild-type tRNA^{Phe} transcript	0.30	(100)	(1.0)
acceptor stem mutants			
A73U	0.41	12	0.088
A73C	0.45	18	0.12
G1A,C72U	0.44	46	0.31
C72U	0.56	27	0.14
variable-pocket mutants			
G20A	1.3	22	0.051
U16C,U17C	0.37	140	1.1
U59C	0.30	110	1.1
C60U	0.29	80	0.82
G53C,C61G	0.27	100	1.1
anticodon stem–loop mutants			
G30C,C40G,A31C,U39G	0.29	40	0.41
A38C	0.57	56	0.30
G34A	0.70	4.4	0.019
A35U	1.6	2.0	0.0038
A36C	0.30	100	1.0
double mutants			
G20A,A73U	3.3	2.3	0.0021
G34A,A73U	1.4	0.28	0.0006

respectively, than that of the wild-type transcript (Table II). These reductions in k_{cat}/K_M are primarily the result of a decreased k_{cat}. Because position 73 is presumed to be single-stranded and both mutants exhibit normal rates of lead cleavage, it is unlikely that the structure of these mutant transcripts is altered. These data confirm the importance of A73 for the specific aminoacylation of tRNA^{Phe} by FRS and suggest that A73 facilitates the catalytic step of the reaction. This is reasonable considering the required close proximity of A73 to the active site of FRS. Similar in vitro kinetic studies have shown that position 73 is important for a number of tRNA synthetase interactions. These include the *E. coli* aspartyl (Hasegawa et al., 1989), alanyl (Shi et al., 1990), glutaminyl (Jahn et al., 1991), valyl (Tamura et al., 1991), and histidyl (Himeno et al., 1989) and the yeast aspartyl-tRNA synthetase (Putz et al., 1991).

A G1–C72 terminal base pair of the acceptor stem, which is adjacent to A73, could also perform a discriminating function. Although most tRNAs in yeast have a G1–C72 pair, tRNAs representing the isoaccepting groups for methionine, aspartate, arginine, lysine, and tyrosine have a different base pair at this position. Two mutants having either an A1–U72 or a G1–U72 base pair were constructed to test the importance of this base-pair for FRS recognition. The G1A,C72U mutant exhibits a 1.5-fold increase in K_M and a 2-fold decrease in k_{cat}, resulting in a 3-fold decrease in k_{cat}/K_M. The C72U mutant is an even poorer substrate for FRS, showing a 6-fold decrease in k_{cat}/K_M. It is not clear whether these relatively modest effects are due to the loss of a sequence-specific contact between the 1–72 base pair and FRS or because these mutations result in a less stable terminal base pair. Whereas fraying of the first base pair in *E. coli* tRNA^{Gln} facilitates the interaction with its cognate synthetase (Rould et al., 1989; Jahn et al., 1991), perhaps for yeast tRNA^{Phe} this situation results in an unfavorable orientation of the 3′ terminus in the active site of FRS. The mutant having a stable C1–G72 base pair was not tested due to the poor ability of T7 RNA polymerase to initiate with pyrimidines (Milligan et al., 1987).

Variable Pocket. The crystal structure of yeast tRNA^{Phe} reveals that the D-loop nucleotides U16, U17, and G20 and the T-loop nucleotides U59 and C60 are not base-paired but are clustered together such that their functional groups are accessible from the back face of the tRNA (Kim et al., 1974a,b; Robertus et al., 1974; Ladner et al., 1975). Klug and co-workers termed this region of the tRNA the "variable pocket" and proposed that these nucleotides would be excellent candidates for sites of sequence-specific contact with synthetases. We have previously shown that G20 is an important recognition nucleotide in yeast tRNA^{Phe} by two criteria. First, the G20U mutation in the yeast tRNA^{Phe} transcript increased K_M 5.5-fold and decreased k_{cat} 2.5-fold (Sampson & Uhlenbeck, 1988) without altering the structure of the tRNA as detected by lead cleavage (Behlen et al., 1990) or NMR (Hall et al., 1989). Second, the U20G mutation in the *E. coli* tRNA^{Phe} background substantially improved its ability to aminoacylate with yeast FRS (Sampson et al., 1989).

To further test the importance of position 20, we constructed a mutant having A20. The G20A mutant exhibits a 4-fold increase in K_M and a 5-fold decrease in k_{cat}, resulting in a 20-fold decrease in k_{cat}/K_M (Table II). The normal rate of lead cleavage observed for this mutant suggests that the effect on aminoacylation is not a result of an altered tertiary structure. These results are comparable to those previously observed for the G20U mutant (Sampson & Uhlenbeck, 1988), clearly indicating that a guanosine at position 20 is required for a proper interaction between tRNA^{Phe} and FRS. Interestingly, G20 is unique to tRNA^{Phe} in all archaebacterial and eukaryotic organisms for which sequence data are available (Sprinzl et al., 1989). Although uniqueness does not necessarily define a recognition site, this association suggests that all archaebacterial and eukaryotic organisms may have conserved the G20 recognition site. Similarly, it has been shown for *E. coli* tRNA^{Arg} that the unique A20 is an important recognition site for its cognate arginyl-tRNA synthetase (Schulman & Pelka, 1989) and contributes substantially to its in vivo identity (McClain & Foss, 1988; McClain et al., 1990).

To test the other four variable-pocket nucleotides in tRNA^{Phe} for potential interaction with FRS, three mutants were constructed. When the two D-loop nucleotides U16 and U17 were changed simultaneously to give the mutant U16C,U17C, there was no detectable effect on either the aminoacylation kinetics or the lead cleavage rate. The two variable-pocket mutants U59C and C60U were also normal substrates for FRS. Thus, there is no indication that FRS interacts specifically with these four nucleotides. This conclusion is supported by the normal aminoacylation kinetics observed for the tRNA$^{Tyr\rightarrow Phe}$ transcript which has both a C59 and a U60 and the tRNA$^{Met\rightarrow Phe}$ transcript which has an A17 (Sampson et al., 1989). However, because the U59C and C60U mutants contained conservative pyrimidine to pyrimidine changes, these data do not rule out the possibility that FRS could discriminate among tRNAs having a purine at these positions.

The G53–C61 base pair, which is located adjacent to the variable-pocket nucleotides U59 and C60, is highly conserved among the nonmitochondrial tRNAs (Sprinzl et al., 1989). Although this base pair could not be used by FRS to discriminate between tRNAs, it could still provide a site of nonspecific contact with the enzyme. To test this possibility, the conserved G53–C61 base pair was inverted. While the G53C,C61G mutant exhibits normal aminoacylation kinetics with FRS, its rate of lead cleavage is reduce by about 10-fold. The crystal structure of the native yeast tRNA^{Phe} reveals a tertiary hydrogen bond between the exocyclic amino group of G53 and the phosphate oxygen of residue 60 (Ladner et al., 1975), suggesting that a G to C substitution at position 53 disrupts this interaction (Behlen et al., 1990). Thus, while a distortion in this region of the tRNA tertiary structure disrupts the Pb^{2+} binding domain, it is not sensed by the synthetase.

These data together strongly suggest that FRS specifically inspects the variable pocket only at position 20.

Anticodon Region. Anticodon loop substitution methods have been used previously to introduce nucleotide changes into the anticodon of native yeast $tRNA^{Phe}$ to test their importance for FRS recognition (Bruce & Uhlenbeck, 1982; Wittenberg & Uhlenbeck, 1985). While changing the universally conserved U33 or the hypermodified nucleotide Y37 had no significant effect on the aminoacylation kinetics with FRS, nucleotide changes in the three anticodon nucleotides G34, A35, and A36 reduced k_{cat}/K_M 2–12-fold depending on the position and the type of substitution made. The introduction of the phenylalanine anticodon nucleotides G34, A35, and A36 into three noncognate yeast tRNA transcripts resulted in good substrates for FRS (Sampson et al., 1989), supporting the contention that FRS interacts specifically with the anticodon loop. These observations prompted the preparation of $tRNA^{Phe}$ transcripts having mutations at the neighboring A38 residue and the A31–U39 and G30–C40 base pairs. In addition, a single mutation at each of the three anticodon positions was made to test whether the effects of these substitutions in the unmodified $tRNA^{Phe}$ are in parallel with those previously obtained in the native $tRNA^{Phe}$ background.

The G30C,C40G,A31C,U39G anticodon stem mutant exhibits a 2-fold lower k_{cat}/K_M due to a decrease in k_{cat} (Table II). The anticodon loop A38C mutant exhibits a 3-fold decrease in k_{cat}/K_M as a result of a small change in both kinetic parameters. While these relatively modest effects on k_{cat}/K_M support the contention that FRS interacts with the anticodon region, it is not clear whether they are due to the loss of sequence-specific contacts or due to an indirect effect. According to the crystal structure of native yeast $tRNA^{Phe}$, an A to C change at position 38 may disrupt the normal hydrogen bond between the O2 of C32 and the N6 amino proton of A38, thereby destabilizing the anticodon loop structure. The nucleotide composition of the anticodon stem could also affect the anticodon loop structure, presumably due to slight changes in base-stacking interactions. Because we observe no significant effect on the lead cleavage rates for the anticodon stem mutants, any structural change is not propagated as far as the lead binding domain.

With the exception of the A36C mutant, the substantial decrease in k_{cat}/K_M for the anticodon mutant transcripts supports the importance of these nucleotides for FRS recognition (Table II). However, the effect of these mutations on both the magnitude of the k_{cat}/K_M value and the relative contribution of each individual parameter is strikingly different from those previously reported for the same mutations in the native $tRNA^{Phe}$ background (Bruce & Uhlenbeck, 1982). In the unmodified $tRNA^{Phe}$ background, the A36C mutation had no effect on either K_M or k_{cat} (Table II) whereas in the native $tRNA^{Phe}$ background, this mutation decreased the k_{cat}/K_M 4-fold as a result of a lowered k_{cat}. The A35U mutation in the unmodified background increased K_M 5-fold and decreased k_{cat} 50-fold, resulting a k_{cat}/K_M 250-fold lower than that of the wild-type $tRNA^{Phe}$ transcript. However, for this same mutation in the native $tRNA^{Phe}$ background, a 4-fold increase in K_M and only a 2-fold decrease in k_{cat} were observed. Finally, the G34A mutation in the unmodified $tRNA^{Phe}$ background reduced the k_{cat}/K_M about 80-fold due primarily to a decreased k_{cat}. This is in contrast to the G34A mutation in the native $tRNA^{Phe}$ background in which only a 12-fold decrease in k_{cat}/K_M was observed due to an increase in K_M.

The normal lead cleavage rates for the G34A and A35U mutant transcripts indicate that these mutations do not affect the overall tertiary folding of the tRNA (Table I). Thus, the large discrepancy between the native and unmodified backgrounds for the same anticodon mutation is most likely the result of a localized anticodon effect due to the absence of the modified nucleotides in this region. Because both the native $tRNA^{Phe}$ mutants and the transcripts lack the hypermodification at position 37, the absence of this modification is not the cause of the discrepancy. In the native $tRNA^{Phe}$, however, position 39 is a pseudouridine which is a highly conserved modification in all tRNAs which have an A31 (Sprinzl et al., 1989). It has been shown that poly(A-Ψ) has a higher melting temperature than that of poly(A-U) (Pochon et al., 1964). Although the effect of pseudouridine on the stabilization of a single internal base pair is minimal (Hall & McLaughlin, 1991), pseudouridine may contribute substantially to the stability of a terminal base pair such as A31–Ψ39. This view is consistent with NMR data for $tRNA^{Phe}$. The H3 imino proton involved in the A31–Ψ39 base pair is clearly observed for the native yeast $tRNA^{Phe}$ (Roy et al., 1982, 1984; Heerschap et al., 1983). For the unmodified $tRNA^{Phe}$ transcript, however, the H3 proton involved in the corresponding A31–U39 base pair is not observed, suggesting that it is much less stable (Hall et al., 1989). Thus, in the absence of Ψ39, additional anticodon mutations may further destabilize the loop structure and displace any remaining anticodon nucleotides relative to the synthetase binding pocket. In this regard, it is interesting that a single mutation in the unmodified background reduces k_{cat}/K_M about the same magnitude predicted for a double anticodon mutant in the native $tRNA^{Phe}$ background. Therefore, caution must be taken when interpreting results of mutations in regions of RNA molecules where nucleotide modifications are normally present.

Double Mutants. The relatively modest contribution of G20, A73, and the individual anticodon nucleotides to k_{cat}/K_M suggests that the overall specificity of FRS for $tRNA^{Phe}$ in vivo could not be achieved by any one of these nucleotides individually. Because these nucleotides are located in three distinct regions of the tRNA, it is reasonable to expect that the required specificity is achieved by the three regions acting together. To test whether the three regions contribute independently to k_{cat}/K_M or show cooperative behavior, double mutants were prepared having a single change in two of the FRS recognition regions simultaneously. Both the G20A,A73U and G34A,A73U double mutants aminoacylated poorly, showing a relative k_{cat}/K_M of 2.1×10^{-3} and 6.0×10^{-4} that of $tRNA^{Phe}$, respectively. If these two regions contribute independently, the predicted k_{cat}/K_M for a double mutant would be calculated as the product of the k_{cat}/K_M values for the individual mutants given in Table II. This would give a predicted k_{cat}/K_M for the G20A,A73U mutant of $(0.051)(0.088) = 4.5 \times 10^{-3}$, which is about 2-fold greater than the measured value. The G34A,A73U mutant gives a predicted value of $(0.019)(0.088) = 1.7 \times 10^{-3}$, which is about 3-fold greater than the measured value. This 2–3-fold difference between the predicted and measured k_{cat}/K_M values is substantially smaller than that of the relative effect of each individual mutation on k_{cat}/K_M, suggesting that these three regions contribute independently. A similar result has been reported for double anticodon mutants in yeast $tRNA^{Tyr}$ (Bare & Uhlenbeck, 1986), suggesting that nucleotides in a single region can contribute independently to the k_{cat}/K_M of aminoacylation.

Predicting the Kinetics of Misacylation for Other tRNAs. The data presented above support our model that the specific recognition of $tRNA^{Phe}$ by FRS is primarily achieved by five

Table III: Relative k_{cat}/K_M Values for Mutations in tRNA^{Phe}

position	recognition sites nucleotide identity			
	U	C	A	G
20	0.071	(0.061)[a]	0.051	1.0
34	0.13	0.088	0.078	1.0
35	0.10	0.12	1.0	(0.11)
36	0.39	0.23	1.0	(0.31)
73	0.088	0.12	1.0	(0.10)

[a] Data in parentheses are the average of the other two mutants.

nucleotides, G20, G34, A35, A36, and A73, with smaller contributions coming from several adjacent nucleotides. Because these five nucleotides appear to contribute independently to the k_{cat}/K_M, it should be possible to predict the k_{cat}/K_M for the misacylation of noncognate tRNAs and compare them to measured values. The accuracy of such a prediction should provide an additional test of our model.

The k_{cat}/K_M values for each of the five recognition sites used to calculate the predicted overall k_{cat}/K_M for a number of tRNAs are given in Table III. The relative k_{cat}/K_M values for nucleotide substitutions at positions 20 and 73 are taken from this work and from Sampson and Uhlenbeck (1988), while those for the three anticodon positions 34, 35, and 36 are taken from Bruce and Uhlenbeck (1982). The k_{cat}/K_M values for the anticodon substitutions in the unmodified background determined in this work were not used because fewer mutations were tested and, as discussed above, the data appear anomalous. For particular nucleotide substitutions where no kinetic data are available, the averages of the measured k_{cat}/K_M values for the other two mutations at that position were used.

Table IV lists 13 tRNAs where the k_{cat}/K_M values of misacylation with FRS have been measured by other investigators. Because the buffer conditions used in the previous studies are similar to those which we have used here, a direct comparison of the relative k_{cat}/K_M values is justified. The measured k_{cat}/K_M for each tRNA in Table IV is normalized to the value obtained for the control yeast tRNA^{Phe} in the given experiment. The predicted k_{cat}/K_M for each of the tRNAs in Table IV was calculated simply by assuming that every substitution of G20, G34, A35, A36, and A73 in the noncognate tRNA decreased k_{cat}/K_M by the same amount measured in the tRNA^{Phe} background. For example, *E. coli* tRNA^{Val1} has G20, U34, A35, C36, and A73, resulting in a calculated k_{cat}/K_M of misacylation by FRS of (1)(0.13)(1)(0.23)(1) = 0.030. In cases where the nucleotide of interest is modified, the k_{cat}/K_M value corresponding to the parental nucleotide in the tRNA gene sequence was used. The presence of nucleotides at positions 20a and 20b in several noncognate tRNAs is not given additional weight, even though they could further reduce the actual k_{cat}/K_M value.

This simple five-nucleotide model is remarkably effective at predicting the k_{cat}/K_M for the 13 tRNAs (Table IV). The phenylalanine tRNAs from *Schizosacchromyces pombe* and wheat germ possess all five of the FRS recognition nucleotides and have a predicted k_{cat}/K_M only slightly lower than the measured value. *E. coli* tRNA^{Phe}, which contains four of the five FRS recognition nucleotides, has a predicted k_{cat}/K_M 2-fold lower than the measured value. This is consistent with our previous observation that an *E. coli* tRNA^{Phe} transcript having a U20G mutation exhibits a measured k_{cat}/K_M 2-fold lower than that of the control yeast tRNA^{Phe} transcript (Sampson et al., 1989). The k_{cat}/K_M for *E. coli* tRNA^{Val1}, which has three of the FRS recognition nucleotides, is predicted the best. The k_{cat}/K_M values for the noncognate tRNAs which have only one or two of the FRS recognition nucleotides

Table IV: Calculated and Measured k_{cat}/K_M Values of Misacylation of tRNAs by FRS

tRNA	nucleotide position					k_{cat}/K_M[a] predicted	k_{cat}/K_M[b] measured
	20	34	35	36	73		
yeast Phe	G	G	A	A	A	(1)	(1)
wheat Phe	G	G	A	A	A	1.0	1.7
S. pombe Phe	G	G	A	A	A	1.0	1.3
E. coli Phe	U	G	A	A	A	0.071	0.029
E. coli Val1	G	U	A	C	A	0.030	0.023
E. coli Ala1	G	U	G	C	A	0.0035	0.0097
E. coli Ala2	G	U	G	C	A	0.0035	0.0042
E. coli Val2	U,U	G	A	C	A	0.014	0.0070
E. coli Met	U,U	C	A	U	A	0.0021	0.0057
yeast Met^M	A	C	A	U	A	0.0018	0.0050
E. coli Ile	U,U	G	A	U	A	0.024	0.0047
E. coli Lys	U	U	U	U	A	0.00038	0.0039
E. coli Gly3	U	G	C	C	U	0.00019	nd[c]
E. coli His	U	G	U	G	C	0.00026	nd

[a] Predicted k_{cat}/K_M values are calculated as the product of the individual relative k_{cat}/K_M values for each recognition site given in Table III. For tRNAs with more than one nucleotide at position 20, k_{cat}/K_M = 0.061 is used. For example, the k_{cat}/K_M of *E. coli* tRNA^{Val2} which has U20, U20a, G34, A35, C36, and A73 is calculated to be (0.061)(1.0)(1.0)(0.23)(1.0) = 1.4×10^{-2}. [b] Measured data from Roe et al. (1973), McCutchan et al. (1978), and Feldmann and Zachau (1977). [c] Not determined.

are also quite accurately predicted. These include the *E. coli* tRNAs Val2, Ala1, Ala2, Met, and yeast Met^M, all of which are predicted within a factor of 3 of the measured value. The *E. coli* tRNAs His and Gly3 are predicted to have the lowest k_{cat}/K_M values, which is consistent with the inability of these tRNAs to be misacylated by FRS (Roe et al., 1973). The two *E. coli* tRNAs Lys and Ile are predicted the least well by this model. The tRNA^{Ile} is unique among the tRNAs in Table IV in that it has an A1–U72 base pair which we have shown here to reduce k_{cat}/K_M about 3-fold. If we include this factor in the predicted k_{cat}/K_M, the discrepancy between the predicted and measured values is reduced substantially. Although it is unclear why the predicted k_{cat}/K_M for tRNA^{Lys} is 10-fold lower than the measured value (Roe et al., 1973), the overall accuracy of these predictions strongly supports not only the importance of the five FRS recognition nucleotides but also their independent contribution to the k_{cat}/K_M of aminoacylation by FRS.

Specificity of FRS in Yeast. To test whether the five-nucleotide recognition model could account for the specificity of FRS for tRNA^{Phe} within yeast, all available yeast tRNA and tRNA gene sequences (Sprinzl et al., 1989) were examined for the identity of the nucleotides at positions 20, 34, 35, 36, and 73, and the data in Table III were used to calculate k_{cat}/K_M values for each one (Table V). It is reassuring to find that, as expected, none of the 19 other isoaccepting groups have all 5 of the FRS recognition nucleotides we identified. For the 17 tRNAs which have none or only 1 of the FRS recognition sites, the k_{cat}/K_M values are predicted to be less than 2.5×10^{-4} of that of tRNA^{Phe}, undoubtably sufficient to ensure specificity. Indeed, misacylation of noncognate *E. coli* tRNAs by the *E. coli* valyl and methionyl synthetases gave similar relative k_{cat}/K_M values (Schulman & Pelka, 1988). However, the 14 yeast tRNAs which have 2 or 3 FRS recognition nucleotides are predicted to be much better substrates for FRS, having a k_{cat}/K_M of from 6.1×10^{-3} to 8.1×10^{-4} that of tRNA^{Phe}. Although the presence of competing cognate synthetases reduces the overall amount of misacylation in vivo (Yarus, 1972; Swanson et al., 1988; Hou & Schimmel, 1989), the question remains whether a difference in k_{cat}/K_M of as little as 170-fold is sufficient to ensure specificity in vivo.

An important clue to the question comes from the data of

Table V: Predicted k_{cat}/K_M for Misacylation of Yeast tRNAs by FRS

tRNA	nucleotide position 20	34	35	36	73	no. of FRS sites	rel k_{cat}/K_M[a]
Phe	G	G	A	A	A	5	(1.0)
Tyr	U,U,U	G	U	A	A	3	6.1×10^{-3}
Leu	U,C,U	C	A	A	A	3	5.4×10^{-3}
Leu	U,U,U	U	A	G	A	2	2.5×10^{-3}
Ile	U,U	U	A	U	A	2	2.5×10^{-3}
Gly	U	G	C	C	A	2	2.1×10^{-3}
Ile	U,U	A	A	U	A	2	1.9×10^{-3}
His	U,U	G	U	G	A	2	1.9×10^{-3}
Met^I	A	C	A	U	A	2	1.8×10^{-3}
Met^M	A	C	A	U	A	2	1.8×10^{-3}
Val	U,U,C	U	A	C	A	2	1.8×10^{-3}
Val	C,U	C	A	C	A	2	1.2×10^{-3}
Val	U,U	A	A	C	A	2	1.1×10^{-3}
Trp	U	C	C	A	A	2	8.1×10^{-4}
Cys	U	G	C	A	U	2	8.1×10^{-4}
Thr	U	A	G	U	A	1	2.4×10^{-4}
Asn	U,U	G	U	U	G	1	2.4×10^{-4}
Gly	U,U	U	C	C	A	1	2.4×10^{-4}
Ala	U	U	G	C	A	1	2.4×10^{-4}
Ala	U	A	G	C	A	1	1.5×10^{-4}
Asp	U,C	G	U	C	G	1	1.4×10^{-4}
Ser	U,U	G	C	U	G	1	3.2×10^{-4}
Ser	U,U	U	G	A	G	1	9.1×10^{-5}
Ser	U,U	C	G	A	G	1	6.2×10^{-5}
Ser	U,U	A	G	A	G	1	5.5×10^{-5}
Arg	C	U	C	U	G	0	4.1×10^{-5}
Arg	U,C	A	C	G	G	0	1.9×10^{-5}
Lys	U	U	U	U	G	0	3.7×10^{-5}
Lys	U	C	U	U	U	0	2.5×10^{-5}
Pro	U	U	G	G	C	0	3.9×10^{-5}
Gln	U,U	U	U	G	U	0	2.2×10^{-5}
Gln	U,U	C	U	G	U	0	1.5×10^{-5}

[a] Calculated using the method described in the legend to Table IV.

Feldmann and Zachau (1977), who found that yeast $tRNA^{Met\text{-}M}$, which contains two FRS recognition sites, can be misacylated with FRS with a relative k_{cat}/K_M of 5.0×10^{-3}, which is in reasonable agreement with the predicted value of 1.8×10^{-3}. Since $tRNA^{Met\text{-}M}$ is not expected to be significantly misacylated by FRS in vivo, it appears that only the seven yeast tRNAs in Table V with higher calculated k_{cat}/K_M values than $tRNA^{Met\text{-}M}$ could be potentially misacylated by FRS.

There are at least two reasons why the remaining seven yeast tRNAs in Table V are likely to be much worse substrates than calculated by the simple five-nucleotide model. First of all, the model does not take into account the fact that many of these tRNAs have a larger D-loop or smaller variable region than $tRNA^{Phe}$. As discussed by Giege et al. (1990), such tRNAs will not position potential FRS recognition nucleotides in such a way to facilitate their interaction with the enzyme. Yeast $tRNA^{Tyr}$ is a good example of such a tRNA. Although it has three FRS recognition nucleotides, its measured k_{cat}/K_M of misacylation is about 50-fold lower than predicted by the five-nucleotide model (Bare & Uhlenbeck, 1985). Indeed many of the 13 nucleotide changes required to convert $tRNA^{Tyr}$ into a good substrate for FRS were needed to correctly position the five recognition nucleotides (Sampson et al., 1989).

Another factor that can lead to an incorrect estimate of the k_{cat}/K_M of misacylation is the presence of negative elements in a noncognate tRNA that override the positive effect of any FRS recognition nucleotide. For example, the extensive contact between FRS and the variable region of $tRNA^{Phe}$ deduced by footprinting studies (Romby et al., 1985) suggests that the large extra stem–loop structure in the two yeast $tRNA^{Leu}$ sequences could sterically block their binding to FRS despite the fact that they contain two or three FRS recognition nucleotides. Similarly, base modifications at one of the FRS recognition sites in noncognate tRNAs could prevent access to FRS, as has been found in other systems (Muramatsu et al., 1988; Perret et al., 1990).

From this analysis, we propose that the specificity of FRS for $tRNA^{Phe}$ in yeast is achieved by making productive contacts with the five major recognition nucleotides, G20, G34, A35, A36, and A73. The noncognate tRNAs in yeast either are missing a sufficient number of recognition nucleotides or have a structure imcompatible with the formation of a productive complex with FRS. Because the competition equilibrium between all tRNAs and synthetases is complex and is well-known to affect specificity (Yarus, 1972; Swanson et al., 1988; Hou & Schimmel, 1989), it would be desirable to test this model in vivo. However, since the anticodon is an important determinant for FRS, the approach involving amber suppressor tRNAs (Normanly et al., 1986; Edwards et al., 1991) will not be appropriate.

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