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Recognition nucleotides for human phenylalanyl-tRNA synthetase

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ABSTRACT

The specificity of the interaction between $tRNA^{Phe}$ and phenylalanyl-tRNA synthetase isolated from human placenta was investigated. Using yeast $tRNA^{Phe}$ transcripts with different point mutations it was shown that all the five recognition points for the yeast phenylalanyl-tRNA synthetase (G_{20}, G_{34}, A_{35}, A_{36} and A_{73}) are also important for the reaction catalyzed by the human enzyme. A set of mutations in nucleotides involved in tertiary interactions of $tRNA^{Phe}$ revealed that mutations which maintained the proper folding of the molecule had almost no influence on the efficiency of aminoacylation. The most striking difference between the yeast and human phenylalanyl-tRNA synthetases involved a mutation in the lower two base pairs of the anticodon stem. This mutation did not affect aminoacylation with the yeast enzyme, but greatly reduced activity with human phenylalanyl-tRNA synthetase.

INTRODUCTION

Two complementary approaches permit the identification of nucleotides participating in the recognition of tRNAs by their cognate synthetases (1). The first utilizes the gene for the amber suppressor derivative of a given tRNA and determines the amino acid inserted at an amber codon of a test protein by protein sequencing (2, 3). The second involves measuring the k_{cat} and K_M for aminocylation of *in vitro* tRNA transcripts using a purified aminoacyl-tRNA synthetase (4–7). In both cases, the important nucleotides are identified by directed mutagenisis of the tRNA gene and confirmed by 'swap' experiments that change other tRNAs into substrates for the synthetase of interest (3, 6, 8–11). The 'recognition set' of nucleotides determined by *in vitro* aminoacylation studies may only be part of the 'identity set' determined by the *in vivo* experiments since it will not include residues necessary to prevent aminoacylation by other synthetases that are present *in vivo*.

It is of considerable interest to determine whether the recognition or identity nucleotides determined for a given synthetase remain the same in other organisms. It has been shown that at least the primary determinant of *E. coli* alanyl-tRNA synthetase, the G_3-U_{70} pair in the acceptor stem, is also required for the B. mori and human enzymes (12). Here we present *in vitro* aminoacylation data for the human phenylalanyl tRNA synthetase (FRS) that permits the proposal of a tentative recognition set. This represents the first such analysis for a mammalian aminoacyl-tRNA synthetase and allows a comparison with similar data obtained with both yeast (11, 13, 14) and *E. coli* (15) FRS. Despite their evolutionary distance, the human, yeast and bacterial phenylalanyl systems shown several similarities. All three enzymes are quite large and have the rare $\alpha_2\beta_2$ quaternary structure (16,17). All three tRNAs are the same length and share most of the same tertiary interactions, suggesting a very similar tertiary folding. Indeed, all 17 positions where the human $tRNA^{Phe}$ sequence differs from yeast $tRNA^{Phe}$ are within base-paired regions (Fig. 1). Conveniently, yeast Phe has been reported to be an excellent substrate for human FRS (17).

MATERIALS AND METHODS

The construction of plasmids containing wild type and mutant yeast $tRNA^{Phe}$ genes directly behind the T7 RNA polymerase promotor are described elsewhere (4, 13). The *in vitro* transcription of these plasmids after cleavage with BstNI endonuclease was carried out as described previously (4). All transcripts contained a 5′ terminal triphosphate instead of the 5′ terminal monophosphate present in $tRNA^{Phe}$. It is not expected that the presence of a 5′ triphosphate affects the kinetic parameters of aminoacylation significantly since little effect has been seen for other tRNA transcripts (4, 15, 18). Unfractionated human tRNA was isolated from human placenta according to Roe (19).

Human FRS was purified from human placenta to a specific activity of 260 units/mg (17). Aminoacylation kinetics at 37° were performed in 60μl reaction mixtures containing 30 mM Hepes-KOH (pH 7.4), 15mM $MgCl_2$, 25mM KCl, 10μM (^{3}H)phenylalanine, 2mM ATP and 4mM DTT. In order to ensure that the conformation was correct, the tRNA samples stored in H_2O were heated to 90° for 2–3 min and cooled to 25°C prior to the addition to the aminoacylation reaction mixture. Preliminary experiments with 1μM tRNA and 0.2 units/ml enzyme were used to confirm that each tRNA mutant could be aminoacylated to greater than 1400 pmoles/A_{260} and thus were

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chemically pure. For those tRNAs that aminoacylated very poorly with human FRS, purity was confirmed with yeast FRS. K_M and k_{cat} values were obtained from an Eadie-Hofstee analysis of the initial rates using six concentrations of tRNA and the appropriate concentration of human FRS. At 20 sec intervals, 7μl aliquots were spotted on Whatman 3MM paper and treated as previously described (20). The correlation coefficients for the Eadie-Hofstee plots from six tRNA concentrations were 0.92 or greater and the values of k_{cat}/K_M can be considered to be within 10% of the indicated values.

RESULTS AND DISCUSSION

As reported previously (17), native yeast $tRNA^{Phe}$ is an active substrate for human FRS. Under the buffer conditions chosen for these experiments, it shows only a two fold higher K_M and a slightly higher k_{cat} than native human $tRNA^{Phe}$ (Table 1). The unmodified yeast $tRNA^{Phe}$ transcript is only a slightly worse substrate than its modified counterpart. Taken together, these results suggest that the large available collection of mutant yeast $tRNA^{Phe}$ transcripts can be used for identification of human FRS recognition nucleotides (4, 11, 13, 14).

Table 1 compares the k_{cat} and K_M values for the wild type yeast $tRNA^{Phe}$ transcript with a variety of mutant transcripts. Many of the mutants have previously been shown not to misfold, based on their ability to cleave with lead (21). Thus any changes in their ability to aminoacylate are likely to be the result of a improper contact with the synthetase. For ease of comparison, the value of k_{cat}/K_M for each mutant is normalized to that of the wild type sequence. Similar data for the same mutants with yeast FRS determined previously are also included in Table 1.

A survey of mutations in residues that are single stranded in the yeast $tRNA^{Phe}$ tertiary structure indicates that the positions important for yeast FRS recognition are also important for human FRS recognition. Thus, changes in G20, G34, A35, A36, and A73 all show substantially reduced rate of aminoacylation, while mutation of U16, U17, U59, and C60 have more modest effects. For most of the mutations, the effect on k_{cat}/K_M is much greater for the human FRS than it is for yeast FRS. For example, mutations in positions 20, 34, 35 and 73 reduced k_{cat}/K_M 4−250 fold with the yeast enzyme while it was not possible to obtain reliable kinetic parameters for the same mutations using the human enzyme, even at 1μM tRNA and 0.08 μM enzyme, which is 25 times more than the enzyme concentration usually used for the kinetic study. The upper limits on k_{cat}/K_M of 10^3 that are reported in Table 1 are based on the apparent rate at the highest convenient enzyme and substrate concentrations.

Nine tRNAs containing single or multiple mutations in nucleotides involved in tertiary interactions were tested for aminoacylation with human FRS. In each case, the nucleotides were changed to common phylogenetic variants that do not alter the folding of the tRNA. In every case, these changes did not substantially affect k_{cat}/K_M thereby indicating that these residues did not form part of the recognition set. It is notable, however, that the A_{26}-G_{44} transcript exhibited a much higher k_{cat} than the wild type substrate.

Based on the similarity in the sequences of yeast and human $tRNA^{Phe}$ (Fig. 1A,B) and the recognition nucleotides identified thus far, it was of great interest to test four 'recognition swap' tRNAs prepared previously (11). Three of these tRNAs (Fig. 1C−E) contain multiple nucleotide changes that convert their sequence from another yeast tRNA to one that has the

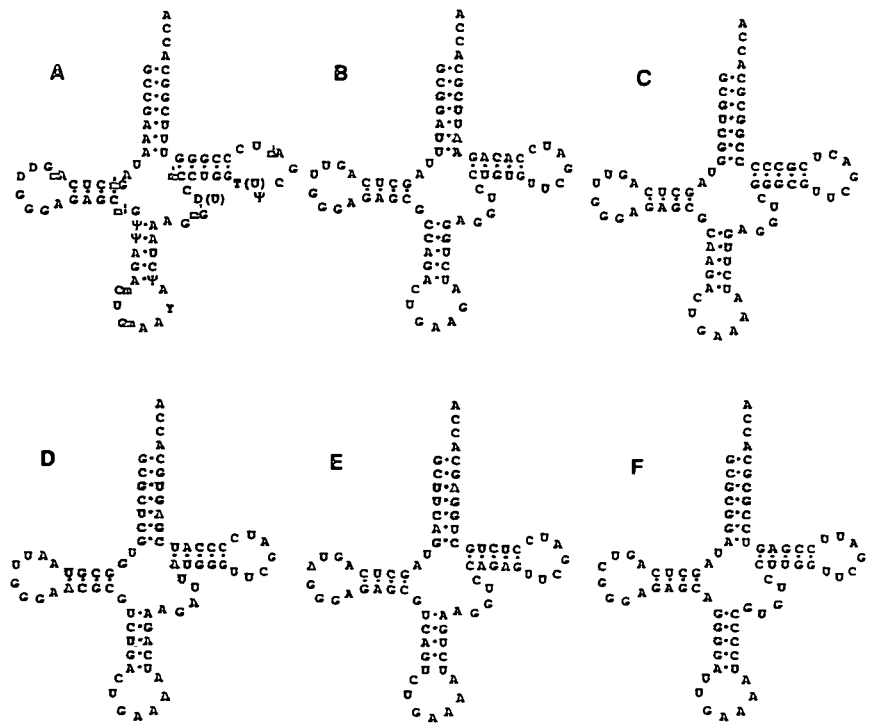

Fig 1. Sequences of (A) native human $tRNA^{Phe}$ (24) and *in vitro* transcripts: (B) Yeast $tRNA^{Phe}$; (C) $tRNA^{Tyr-Phe}$; (D) $tRNA^{Arg-Phe}$; (E) $tRNA^{Met-Phe}$; (F) *E. coli* $tRNA^{Phe}$ [U20G]. The nucleotides in the transcripts which differ from the human $tRNA^{Phe}$ sequence are shaded.

correct number of nucleotides and all the recognition elements needed for yeast FRS. The fourth (Fig. 1F) is *E. coli* $tRNA^{Phe}$ (U20G) which is also a good yeast FRS substrate. Since the human FRS recognition nucleotides identified thus far are the same as those for yeast FRS, all four of these tRNAs would be expected to be good substrates for the human enzyme. As shown in Table 1, both the $tRNA^{Tyr-Phe}$ and the $tRNA^{Arg-Phe}$ transcripts are indeed good substrates, but the *E. coli* $tRNA^{Phe}$ (U20G) shows a somewhat lower k_{cat}/K_M and the $tRNA^{Met-Phe}$ sequence shows very poor aminoacylation. This pattern contrasts with yeast FRS where all four tRNAs are excellent substrates and therefore suggests that the recognition properties of the human enzyme may be different.

Of the 19 residues in the $tRNA^{Met-Phe}$ sequence which differ from human $tRNA^{Phe}$, 16 are base pair substitutions at positions that do not appear to be important based on the activity of yeast $tRNA^{Phe}$, $tRNA^{Tyr-Phe}$ and $tRNA^{Arg-Phe}$ (Fig. 1). Of the remaining three positions that differ (A37, A17 and U31), postion 37 is an A in the active $tRNA^{Tyr-Phe}$ and position 17 is active as either a D (native $tRNA^{Phe}$) or a C (Table 1) and therefore is likely to be active when it is an A. This focuses attention on the unusual U31-U39 pair as a possible reason that $tRNA^{Met-Phe}$ aminoacylates poorly. In order to test whether the lower base pairs in the anticodon stem affect aminoacylation, the G30-C40, A31-U39 pairs were changed to C30-G40, C31-G39. As shown in Table 1, this quadruple mutant remained active with yeast FRS, but was virtually inactive with human FRS. This strongly suggests that one or both of these base pairs are part of the human $tRNA^{Phe}$ recognition set. It is unclear, however, whether the quadruple mutation disrupts a direct contact between enzyme and tRNA or acts indirectly by altering the conformation of the very critical anticodon nucleotides. Effects of the anticodon stem sequence on codon structure and activity are well documented (22,23).

TABLE 1

Aminoacylation of the tRNAs with Human and Yeast FRS

	K_M (nM)	k_{cat} (min^{-1})	Normalized k_{cat}/K_M Human FRS	Yeast FRS†
Modified tRNAs·				
Human $tRNA^{Phe}$ (1A)	60	3 9	4 2	—
Yeast $tRNA^{Phe}$ (1B)	140	4 1	1 9	4.8
Yeast $tRNA^{Phe}$ transcripts				
wild type	220	3.4	(1.0)	(1.0)
G20U	—	—	<0 001	0.083
G34A	—	—	<0 001	0 018
A35U	—	—	<0.001	0.0038
A36C	580	1.1	0.12	1.0
A73C	—	—	<0 001	0 12
A73U	—	—	<0 001	0.088
U59C	360	3.3	0 59	1.1
C60U	570	2.5	0.28	0 82
U16C, U17C	150	1.8	0.78	1.1
G15A, C48U	200	5.0	1 6	1.6
A9G, A23C, U12G	300	3.2	0.63	0 55
C13U, G22A, G46A	220	3.6	1 1	1 2
G10C, C25G	240	1.8	0 49	0.88
G45U	300	3.4	0.73	0 95
A44G	540	3.4	0 41	1.6
A44U	260	3.5	0 87	1.3
G26A	770	5.2	0.44	1.2
G26A, A44G	980	15.1	1.0	1.5
Tyr—Phe	310	1.8	0.38	1.5
Met—Phe	—	—	<0 02	0.68
Arg—Phe	250	3.5	0 91	0.64
E coli $tRNA^{Phe}$ (G20)	1300	1.4	0 07	0.52
G30C, C40G, A31C, U39G	—	—	<0.001	0.41

†Data taken from references 4, 11, 13, 14.

The 12-fold lower k_{cat}/K_M for *E. coli* $tRNA^{Phe}$ (U20G) is somewhat more difficult to explain. Of the 23 nucleotides that differ from the human $tRNA^{Phe}$ sequence, all but U32 can be eliminated by earlier data. Perhaps the U32-A38 base pair can form and disturb the critical anticodon-enzyme interaction. An alternative explanation is based on the recent observation that all mutations at position 20 of *E. coli* $tRNA^{Phe}$ appear to misfold, based on a reduced rate of lead cleavage (15). Although yeast FRS does not seem very sensitive to this, human FRS may be more so. Thus, it is possible that the poor aminoacylation of *E. coli* $tRNA^{Phe}$ U20G is the result of the introduction of disruptive features and not the absence of recognition nucleotides.

Figure 2 summarizes our data defining a tentative recognition set for human FRS. The sixteen circled residues can be eliminated because they are conserved in virtually all cytoplasmic mammalian tRNAs. At the 44 positions indicated by dots, the residue in human $tRNA^{Phe}$ has been changed to one, two, or three different residues in tRNAs described here without altering k_{cat}/K_M. Although we tentatively eliminate these positions as potential recognition nucleotides, it remains possible that they can contribute through a contact with the enzyme that involves a functional group that was not changed. For example, a contact with a purine N7 will not be revealed by an A to G substitution. Of the remaining 17 positions, five single stranded residues have clearly been shown to be recognition nucleotides (G20, G34, A35, A36, A73) and one or both of the lower two base pairs in the anticodon (G30-C40, A31-U39) appear to be important. Of the remaining untested positions, the anticodon nucleotides C32 and A38 are potentially important based on the importance of nearby anticodon residues. Since the G1-C72 pair has a small effect with yeast FRS and is adjacent to the essential A73, it may be important for the human enzyme as well. Indeed, the C72U mutant containing an alteration

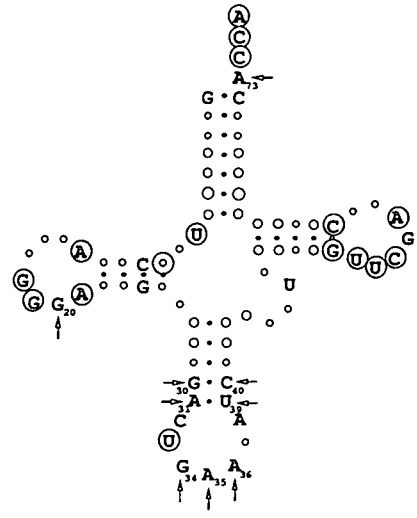

Fig 2. Nucleotides recognized by human FRS. The conserved nucleotides are circled and the small, medium, and large dots indicate positions where the nucleotide in human $tRNA^{Phe}$ has been changed to one, two or three other nucleotides respectively without altering k_{cat}/K_M of aminoacylation. Arrows indicate proposed recognition nucleotides. Remaining residues are untested.

at one nucleotide of the basepair, exhibits a 10-fold reduction in catalytic efficiency (unpublished results). The remaining untested positions (C11, G24, U47 and G57) are unlikely to be important since these nucleotides are at least semi-conserved among known eukaryotic elongator tRNAs (24).

The emerging human FRS recognition set therefore closely resembles that of yeast FRS. The major difference in addition to the quantitative effects of the mutations on the aminoacylation kinetics is that one or two base pairs in the anticodon stem are also included. Since all eukaryotic phenylalanine tRNAs sequenced to date contain the same residues in all nine recognition positions (24), it is possible that they all have very similar recognition sets. In contrast, the *E. coli* FRS recognition set is somewhat different (15). While positions 20, 34, 35, 36 and 73 are also important, residues involved in tertiary interactions [10-25-45 and 26-44] and an additional residue in the variable pocket [59] are also required. In addition, the recognition nucleotide at position 20 is a U instead of a G. Nevertheless, since the same regions of the tRNA are involved, the differences between bacteria and eukaryotes may only reflect small changes in the details of the RNA protein interaction rather than a large change in how the macromolecules interact. Thus, phenylalanyl-tRNA synthetases appear to recognize their cognate tRNA in a similar way.

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