Sequences Outside Recognition Sets Are Not Neutral for tRNA Aminoacylation

EVIDENCE FOR NONPERMISSIVE COMBINATIONS OF NUCLEOTIDES IN THE ACCEPTOR STEM OF YEAST tRNA^{Phe}

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Phenylalanine identity of yeast tRNA^{Phe} is governed by five nucleotides including residues A73, G20, and the three anticodon nucleotides (Sampson et al., 1989, Science 243, 1363–1366). Analysis of in vitro transcripts derived from yeast tRNA^{Phe} and Escherichia coli tRNA^{Ala} bearing these recognition elements shows that phenylalanyl-tRNA synthetase is sensitive to additional nucleotides within the acceptor stem. Insertion of G2-C71 has dramatic negative effects in both tRNA frameworks. These effects become compensated by a second-site mutation, the insertion of the wobble G3-U70 pair, which by itself has no effect on phenylalanylation. From a mechanistic point of view, the G2-C71/G3-U70 combination is not a “classical” recognition element since its antiderminent effect is compensated for by a second-site mutation.

This enlarges our understanding of tRNA identity that appears not only to be the outcome of a combination of positive and negative signals forming the so-called recognition/identity set but that is also based on the presence of nonrandom combinations of sequences elsewhere in tRNA. These sequences, we name “permissive elements,” are retained by evolution so that they do not hinder aminoacylation. Likely, no nucleotide within a tRNA is of random nature but has been selected so that a tRNA can fulfill all its functions efficiently.

The specificity of transfer RNA aminoacylation is a crucial step in protein synthesis. Investigations during the last years have shown that the aminoacylation identity of a tRNA is linked to the presence of specific sets of signals allowing both discrimination by cognate aminoacyl-tRNA synthetases (aaRSs),1 the positive elements, and rejection by noncognate synthetases, the negative elements or antideterminants (1–3). The completeness of a set of positive elements has generally been tested by co-transplantation of the corresponding nucleotides into one or several noncognate host tRNAs that acquire the new aminoacylation properties. In several instances, this approach allowed detection of special requirements for the optimal expression of a given aminoacylation identity set within a host tRNA. Thus, minor elements and conformational features were shown to contribute to aminoacylation identities (e.g. Refs. 4–8).

Recognition elements required for phenylalanylation of yeast tRNA^{Phe} were defined in the pioneering work of Uhlenbeck and co-workers (9, 10) as a set of five major elements. These elements correspond to G20, G34, A35, A36, and A73, and their competence to confer phenylalanine (Phe) identity was first demonstrated by transplantation into four host tRNAs that all acquired optimal phenylalanylation capacities (10). Nucleotides involved in tertiary interactions were shown not to contribute to identity by a direct effect (11). Alternatively, expression of Phe identity in the yeast tRNA^{Ala} context has revealed that PheRS is sensitive to fine local structural features, such as the D-loop and variable region structures (12). Finally, neither study based on sequence comparisons of natural or engineered Phe accepting species, nucleotides within the acceptor stem helix were found important for specificity.

In a previous work, we have been able to create a chimeric tRNA, efficiently recognized and aminoacylated at once by three different aminoacyl-tRNA synthetases including yeast PheRS (6), and found that effective phenylalanylation of this tRNA was dependent, among other features, on the sequence of base pair 2–71 within the acceptor stem. Engineering of a tRNA with multiple specificities was based on the synthesis of a chimeric tRNA^{Ala} containing the recognition sets for yeast PheRS (the five residues listed above), for Escherichia coli AlaRS (the G3-U70 base pair, e.g. Refs. 13 and 14), and for yeast ValRS (A73 and A35; Ref. 15). Notice that the valine identity residues A73 and A35 are common to the Phe recognition set. Simultaneous optimization of alanylation and phenylalanylation efficiencies could be achieved by insertion of specific structural features (the length of the α- and β-domains within the D-loop shaped to 4 and 2 nucleotides and the length of the variable region extended to 5 nucleotides) and mutation of base pair 2–71 in the amino acid acceptor stem from C-G to G-C (6).

Whereas the structural changes introduced were directed by already established yeast PheRS requirements (11, 12), replacement of base pair C2-G71 by G2-C71 in the chimeric tRNA^{Ala} transcript was guided by our present understanding of E. coli tRNA^{Ala} identity. Indeed, this base pair is important.
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**RESULTS**

**Influence of Base Pair G3-U70 on Phenylalanylation within the Yeast tRNA_{Asp} Context**—All experiments performed in our previous work (6) leading to the discovery of a positive effect of a G2-C71 base pair on phenylalanylation were done in the tRNA_{Asp} context. These transcripts contained systematically a G3-U70 base pair, necessary for an efficient concomitant alanylalanylation (6). Influence of this last base pair on the chimeric tRNA_{Phe} transcript, as demonstrated by structural mapping of the RNAs by lead according to procedures described previously (23, 24).

**Aminoacylation Reactions**—Aminoacylation reactions of transcripts derived from tRNA_{Asp} have been performed as described (6) in a medium containing 25 mM Tris-HCl, pH 7.5, 7.5 mM MgCl_2, 0.5 mM ATP, 0.1 mg/ml bovine serum albumin, 50 μM [3H]-labeled phenylalanine, and adequate amounts of tRNA transcript and yeast PheRS. Aminoacylation reactions of transcripts derived from tRNA_{Phe} and tRNA_{Ala} have been performed in a medium containing 30 mM HEPES, pH 7.4, 15 mM MgCl_2, 12 mM ATP, 30 mM KCl, 4 mM DTE, 50 μM [3H]-labeled phenylalanine, tRNA transcript, and yeast PheRS (10). Before aminoacylation, transcripts were renatured by heating at 65 °C for 90 s and slow cooling to room temperature. Assays were performed in the conventional way (25) with incubation at 30 °C. The kinetic constants were determined from Lineweaver-Burk plots. Since the concentration of amino acids is subsaturating, only apparent kinetic parameters are given. They represent an average of at least two independent experiments. Functional properties of mutants are expressed as catalytic efficiencies of phenylalanylation by k_{cat} over K_m ratios. For easier comparisons, these ratios are also normalized with regard to the wild-type molecule.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized on an Applied Biosystems 381 DNA synthesizer using the phosphoramidite method and purified by HPLC on a Nucleosil 125–5-C18 column (Bischoff Chromatography, Zymark-France, Paris). l-[3H] phenylalanine (9.6 × 10^11 Bq/mol) was from Amersham France (Les Ulis). Yeast PheRS was a gift of M. Baltzinger (Strasbourg). T7 RNA polymerase was purified according to method as described previously (19). Restriction enzymes (BstNI, HindIII, and BamHI) and T4 polynucleotide kinase were from New England Biolabs (Beverly, MA). T4 DNA ligase was from Boehringer Mannheim (Meylan, France).

**Cloning and in Vitro Transcription**—All tRNAs used in this work have been obtained by in vitro transcription of synthetic genes. Each of these genes corresponds to the T7 RNA polymerase promoter region directly upstream of the tRNA sequence. The tRNA genes were constructed and cloned into plasmid pUC 119 linearized at dIII sites according to established methods (20). Tg1 cells were transformed. A BstNI site coincidental with the 3′-end of the tRNA sequences allows synthesis of tRNAs ending with the expected CCA sequence. Experimental procedures were described previously (20). In vitro preparation and purification of transcripts was performed according to established procedures (6). Concentration of stock solutions of transcripts have been determined by absorbency measurements at 280 nm. The primary structure of variants has been checked, in particular for the 5′-end G-rich regions where the sequences were carefully verified by appropriate sequencing methods (21, 22). The global folding of the variant transcripts was shown to be the same as that of wild-type tRNA_{Phe} transcript, as demonstrated by structural mapping of the RNAs by lead according to procedures described previously (23, 24).

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**RESULTS**

Influence of Base Pair G3-U70 on Phenylalanylation within the Yeast tRNA_{Asp} Context—All experiments performed in our previous work (6) leading to the discovery of a positive effect of a G2-C71 base pair on phenylalanylation were done in the tRNA_{Asp} context. These transcripts contained systematically a G3-U70 base pair, necessary for an efficient concomitant alanylalanylation (6). Influence of this last base pair on the chimeric tRNA_{Phe} transcript, as demonstrated by structural mapping of the RNAs by lead according to procedures described previously (23, 24).

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Influence of Base Pairs 2–71 and 3–70 on Phenylalanylation within the Yeast tRNA^Phe Context—In order to study the role of base pairs 2–71 and 3–70 on phenylalanylation in the cognate natural tRNA framework, a series of mutants of yeast tRNA^Phe presenting variations in their sequence have been prepared by in vitro transcription (Fig. 1). The wild-type tRNA^Phe transcript presents the sequences C2-G71 and G3-C70, and is referred as molecule D in this work (Fig. 1). Four mutants were designed. Variants E and F differ from wild-type tRNAPhe only at the third base pair by the presence of a G-U pair. This dramatic effect is observed by inversion of base pair 2–71 by a factor 21 (Table 1). Invariant J, a tRNA^Ala with the Phe recognition set and the same sequence as wild-type tRNAPhe at positions 2–71 and 3–70, is 85-fold less well aminoacylated than the reference tRNA^Phe transcript. Both $k_{cat}$ and $K_m$ are about 10-fold lower. Interestingly, in variant J, as in tRNAPhe, a G2-C71/G3-C70 combination of nucleotides has a dramatic negative consequence on phenylalanylation with a loss in aminoacylation efficiency of 2100-fold.

Finally, sequence combinations C2-G71/G3-U70 or G2-C71/G3-C70 confer a free energy of $7.1 \text{ kcal/mol}$, whereas G2-C71/G3-C70 confers only $0.4 \text{ kcal/mol}$, as is the case in the tRNA^Phe context (variant H).

RNA Helix Stabilities—Calculation of thermodynamic stabilities of RNA helices was according to Turner et al. (26). In the presence of a G3-C70 pair, the free energy of the tRNA^Phe accepting helix is between 2 and $3 \text{ kcal/mol}$ lower than in the presence of G3-U70. Indeed, in the case of the tRNA^Phe framework, the combination G2-C71/G3-C70 confers a free energy of $-7.1 \text{ kcal/mol}$, whereas G2-C71/G3-U70 confers only $-5.4 \text{ kcal/mol}$. Similarly, in the tRNA^Ala framework, a G-C/G-U combination leads to $-8.7 \text{ kcal/mol}$, whereas a G-C/G-U combination leads only to $-5.7 \text{ kcal/mol}$.

DISCUSSION

General Considerations—First, we recall that the tRNAs investigated here possess the foreseen sequences and fold correctly as verified by sequence analysis and structural mapping with lead (data not shown). Further, these molecules, aside from containing the Phe recognition elements, possess all the same sequences at their amino acid-accepting extremities, namely a G1-C72 base pair extended by a 3’ A73CCA-end. In what follows, and for the sake of simplicity, we assume that the test transcripts are displayed in Fig. 1. Note that these transcripts share the same fine structural characteristics as tRNA^Phe in terms of d-loop and variable region organizations, namely the same length of the $\alpha$ and $\beta$ regions in the d-loop and the same length of the variable region ($v = 5$). Since constant G18 and G19 residues in the d-loop make long range interactions with the T-loop, in particular the G19-C56 Watson-Crick pair, and that variable region residues are structurally related with d-loop and stem residues (e.g. the G15-C48 Levitt pair and the C13-G22-G46 triple), it can be concluded that the core of both tRNA^Phe and tRNA^Ala transcripts are similar and consequently that the two tRNAs present the same overall three-dimensional structure (1, 6). Variant J, a tRNA^Ala with the Phe recognition set and the same sequence as wild-type tRNAPhe at positions 2–71 and 3–70, is 85-fold less well aminoacylated than the reference tRNA^Phe transcript. Both $k_{cat}$ and $K_m$ are about 10-fold lower. Interestingly, in variant J, as in tRNAPhe, a G2-C71/G3-C70 combination of nucleotides has a dramatic negative consequence on phenylalanylation with a loss in aminoacylation efficiency of 2100-fold.

Finally, sequence combinations C2-G71/G3-U70 or G2-C71/G3-U70 lead to efficient phenylalanylation of tRNA^Ala-derived tRNAs (L = 1 and L = 14, respectively, for variants K and L), as is the case in the tRNA^Phe context (variant H).

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Numerical values used for computing normalized kinetic parameters are taken from Table I. According to the definitions given in Table I, $L = \frac{(k_{cat})_{mutant}}{(k_{cat})_{wt}} \times \frac{(K_m)_{mutant}}{(K_m)_{wt}}$, which can be rearranged as $L = \frac{(k_{cat})_{mutant}}{(k_{cat})_{wt}} \times \frac{(K_m)_{mutant}}{(K_m)_{wt}}$. For a given mutant, $k_{cat}$ and $K_m$ values are normalized for $L = 1$, so that $L_{wt} = \frac{(k_{cat})_{wt}}{(k_{cat})_{mutant}} \times \frac{(K_m)_{wt}}{(K_m)_{mutant}}$. Thus $L_{mutant} = \frac{(k_{cat})_{mutant}}{(k_{cat})_{wt}} \times \frac{(K_m)_{mutant}}{(K_m)_{wt}}$. For variants with $(k_{cat})_{wt} > 1$, the relative contribution of $k_{cat}$ to $L$ is preponderant (these variants have impaired $k_{cat}$ values as compared to wt tRNA); conversely for variants with $(K_m)_{wt} > 1$, the relative contribution of $K_m$ is most important (these variants have a decreased affinity for PheRS as compared to wt tRNA). If for a tRNA variant $(k_{cat})_{N} \sim (K_m)_{N} \sim 1$, the relative contribution of both parameters to $L$ is the same as for wt tRNA. Normalized $k_{cat}$ and $K_m$ values significantly above 1 are emphasized in bold. For complete sequence of tRNA variants, see Fig. 1, wt, wild-type.

| Name of variants & base pairs 2–71/3–70 | L (fold) | Normalized values of $k_{cat}$ and $K_m$ for $L = 1$ |
|-----------------------------------------|---------|--------------------------------------------------|
| Phe                                    |         | $(k_{cat})_N$ $(K_m)_N$ |
| Phe                                    |         | 1.00 1.00 |
| Ala                                    |         | 0.29 2.59 |
| Phe                                    |         | 1.14 0.88 |
| Ala                                    |         | 0.85 1.19 |
| Phe                                    |         | 0.37 2.70 |
| Ala                                    |         | 0.36 2.81 |
| Phe                                    |         | 0.24 0.49 |
| Ala                                    |         | 0.57 0.48 |
| Phe                                    |         | 1.00 0.92 |
| Ala                                    |         | 0.70 0.97 |
| Phe                                    |         | 1.10 0.92 |
| Ala                                    |         | 0.47 0.47 |
| Phe                                    |         | 2.14 0.47 |
| Phe                                    |         | 1.93 0.51 |
| Ala                                    |         | 2.50 0.40 |

variations in their phenylalanylation properties are essentially due to the nucleotide combinations at positions 2–71 and 3–70, although we are aware that additional effects can occur (see below). To facilitate discussion, data are summarized in Table II. Variants are classified as a function of their decreasing ability to be phenylalanylated (increasing $L$-values) and are divided into six groups (a to f) on the basis of the nature of their 2–71 and 3–70 base pairs, and individual kinetic parameters are normalized so that relative contributions of $k_{cat}$ and $K_m$ to $L$ can be easily compared. Several features become immediately apparent. Similar $L$-values can result from different combinations of $k_{cat}$ and $K_m$. This is for instance, the case when comparing the phenylalanylation capacities of variants H and K. The normalized $k_{cat}$ and $K_m$ show that efficient variants ($L < 5$) behave phenomenologically either as wild-type tRNA$^{Phe}$ (variants K and E) or are charged in a mechanism which their binding to PheRS is decreased (normalized $K_m < 1$). Thus $k_{cat}$ is the most important (these variants have a decreased affinity for PheRS as compared to wt tRNA). If for a tRNA variant $(k_{cat})_N \sim (K_m)_N \sim 1$, the relative contribution of both parameters to $L$ is the same as for wt tRNA. Normalized $k_{cat}$ and $K_m$ values significantly above 1 are emphasized in bold. For complete sequence of tRNA variants, see Fig. 1, wt, wild-type.

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According to the current view, tRNA aminoacylations are essentially due to the nucleotide combinations at positions 2–71 and 3–70, although we are aware that additional effects can occur (see below). To facilitate discussion, data are summarized in Table II. Variants are classified as a function of their decreasing ability to be phenylalanylated (increasing $L$-values) and are divided into six groups (a to f) on the basis of the nature of their 2–71 and 3–70 base pairs, and individual kinetic parameters are normalized so that relative contributions of $k_{cat}$ and $K_m$ to $L$ can be easily compared. Several features become immediately apparent. Similar $L$-values can result from different combinations of $k_{cat}$ and $K_m$. This is for instance, the case when comparing the phenylalanylation capacities of variants H and K. The normalized $k_{cat}$ and $K_m$ show that efficient variants ($L < 5$) behave phenomenologically either as wild-type tRNA$^{Phe}$ (variants K and E) or are charged in a mechanism which their binding to PheRS is decreased (normalized $K_m < 1$). Thus $k_{cat}$ is the most important (these variants have a decreased affinity for PheRS as compared to wt tRNA). If for a tRNA variant $(k_{cat})_N \sim (K_m)_N \sim 1$, the relative contribution of both parameters to $L$ is the same as for wt tRNA. Normalized $k_{cat}$ and $K_m$ values significantly above 1 are emphasized in bold. For complete sequence of tRNA variants, see Fig. 1, wt, wild-type.

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pair that decrease the stability of the acceptor stem in comparison with a stem with standard Watson-Crick pairings. We recall that G-U base pairs are important in protein-RNA interactions in general, and in tRNA recognition in particular. For instance, the G3-U70 pair in tRNA-Ala is the major alanine identity element for AlaRS and in E. coli is involved in a subtle recognition process by the synthetase (4, 29–32). In addition to the presence of the exocyclic amino group in the minor groove of the RNA helix, a G-U pair decreases the helix stability as compared with its G-C equivalent since it contains only two hydrogen bonds (33–35). Unlike a G-C pair, a G-U pair was shown, by NMR, to induce a variation in the local helix geometry (36–38). For example, in an RNA helix mimicking the tRNA-Ala acceptor stem, the G-U pair displaces nucleotide C71 and reduces the stacking of the four unpaired nucleotides at the 3’-extremity of the helix (37). The yeast tRNAAsp anticodon helix is another example where a noncanonical G-U pair plays an role in the interaction process with its cognate synthetase by destabilizing an RNA helix. In this case, the G30-U40 pair was shown to be the site of a kink (39) that allows adaptation of the anticodon nucleotides with the AspRS anticodon binding domain (40). Within these lines, we suggest that introduction of a G3-U70 pair into tRNAs inactive for phenylalaninylation brings sufficient flexibility into the acceptor stem to allow a fruitful adaptation of the tRNA to PheRS. Similar interpretation has been given to explain the role of G3–U70 in Ala identity adaptation of the tRNA to PheRS. Similar interpretation has been given to explain the role of G3–U70 in Ala identity adaptation of the tRNA to PheRS.

In conclusion, yeast PheRS can accommodate well alternate combinations of base pairs at positions 2–71 and 3–70 that act as a “permissive box” when G-C/G-U or as a strong “non-permissive box” when G-C/G-C for PheRS recognition. There is a previous example of a functional box in a tRNA amino acid-accepting domain that contains a G-U wobble pair surrounded by two G-C pairs. In this case, however, the G-U pair is part of an “antideterminant box” accounting for nonrecognition of E. coli tRNASec by EF-Tu (44). Considering these facts, it is not excluded that other base pair combinations at positions 2–71 and 3–70 in tRNA-Phe, and more generally elsewhere in this structure, present a non-permissive character or are permissive for PheRS recognition. A systematic search of such combinations will require combinatorial techniques. Altogether, the present findings call for an extended vocabulary for elements involved in tRNA identity. When considering consensus sequences, one has to keep in mind that some nucleotide combinations within “neutral” individual positions might become nonpermissive sequences for some functions of the tRNA. This enlarges tremendously the structural possibility that tRNA-synthetase systems have to ensure their specificity. Moreover, our results suggest that nucleotides within a tRNA have been selected by evolution in such a way that the tRNA can fulfill all its functions in an efficient way. Thus, systematic search of permissive and non-permissive sequence elements is encouraged, not only in the Phe system, but also in aminoacylation systems of other specificities. Knowledge of such elements would help to understand the relationships between tRNA identities for synthetases and their coupled evolution with that of other identities for other proteins recognizing tRNAs.

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Sequences Outside Recognition Sets Are Not Neutral for tRNA Aminoacylation: EVIDENCE FOR NONPERMISSIVE COMBINATIONS OF NUCLEOTIDES IN THE ACCEPTOR STEM OF YEAST tRNA\textsubscript{Phe}
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