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Mutants of *Escherichia coli* initiator tRNA that suppress amber codons in *Saccharomyces cerevisiae* and are aminoacylated with tyrosine by yeast extracts

(aminoacyl-tRNA synthetase recognition/amber suppression/yeast tyrosyl-tRNA synthetase)

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ABSTRACT **We recently described mutants of *Escherichia coli* initiator tRNA that suppress amber termination codons (UAG) in *E. coli*. These mutants have changes in the anticodon sequence (CAU → CUA) that allow them to read the amber codon and changes in the acceptor stem that allow them to bind to the ribosomal aminoacyl (A) site. We show here that a subset of these mutants suppress amber codons in *Saccharomyces cerevisiae* and that they are aminoacylated with tyrosine by yeast extracts. Analysis of a number of mutants as substrates for yeast tyrosyl-tRNA synthetase has led to identification of the C1·G72 base pair and the discriminator base A73, conserved in all eukaryotic cytoplasmic and archaebacterial tyrosine tRNAs, as being important for recognition. Our results suggest that the C1·G72 base pair and the discriminator base, in addition to the anticodon nucleotides previously identified [Bare, L. A. & Uhlenbeck, O. C. (1986) *Biochemistry* 25, 5825–5830] as important in yeast tyrosyl-tRNA synthetase recognition, may comprise the critical identity determinants in yeast tyrosine tRNA.**

The ability to introduce site-specific mutations into tRNA genes (1–4) and to study the properties of mutant tRNAs *in vitro* and *in vivo* has led to major advances in identification of sequence elements important in recognition of tRNAs by the corresponding aminoacyl-tRNA synthetases. These studies (reviewed in refs. 5–7) show that, in most cases, sequences important for specificity in recognition are located primarily in the anticodon sequence (8–10), the acceptor stem (11, 12), or both (13–16). However, there are several examples in which nucleotides important for recognition are more widely dispersed, including a combination of anticodon sequence, acceptor stem, dihydrouridine stem, dihydrouridine loop, variable loop, etc. (4, 17–19). Details of the nature of interactions between aminoacyl-tRNA synthetases and tRNAs are beginning to emerge from parallel work on x-ray crystallographic structure of the enzyme–tRNA complexes (13, 15, 20). In the two known structures of such complexes (15, 20), the enzyme makes extensive contact with the tRNA substrate and there is substantial change in the local structure of the tRNA in the anticodon loop and in the acceptor stem. Whether this will prove to be the case for the other aminoacyl-tRNA synthetase/tRNA combinations, in which the recognition elements are located exclusively in the acceptor stem, is not known (11, 12).

With a few exceptions (17, 18, 20–22), recent work on aminoacyl-tRNA synthetase recognition of tRNAs has focused on enzymes from *Escherichia coli*. Here, we describe studies on recognition of tRNAs by yeast tyrosyl-tRNA synthetase (TyrRS). For studies of structure–function relationships of *E. coli* initiator tRNA, we described previously the generation and characterization of mutants that suppress amber codons in *E. coli* (16, 23). One of these mutants has a C1·G72 base pair, at the end of the acceptor stem. This feature is otherwise found only in tyrosine tRNA of eukaryotic cells and archaebacteria and in proline tRNAs of eubacteria and bacterial viruses (24). We therefore investigated the possibility that this mutant might function as an amber suppressor in the yeast *Saccharomyces cerevisiae*. We show here that it does and that it is aminoacylated with tyrosine by yeast extracts. Results of aminoacylation assays on several of the mutants suggest an absolute requirement for the C1·G72 base pair for aminoacylation with tyrosine. Comparison of kinetic parameters in aminoacylation suggests that one of the mutant *E. coli* initiator tRNAs is as good a substrate for yeast TyrRS as a yeast tyrosine-inserting amber suppressor tRNA studied by Bare and Uhlenbeck (21). We discuss the implications of these results for recognition of tRNAs by yeast TyrRS.

MATERIALS AND METHODS

Yeast Strain, Plasmids, and Extract. *S. cerevisiae* HEY301-129 (*a met8-1 trp1-1 his4-580 leu2-3,112 ura3-1 ade1 can1-100*) was from H. Edwards (25) and plasmid YEP420 was from D. Botstein (Stanford University). Constructs for expression of mutant *E. coli* initiator tRNA genes in *S. cerevisiae* contained an ≈450-base-pair (bp) DNA fragment (26) cloned into the *Hin*dIII–*Bam*HI site of YEP420. Yeast extracts containing RNA polymerase III and aminoacyl-tRNA synthetase activity were provided by T. A. Weil (27).

Yeast Genetic Methods and Transformation. Standard techniques for growth and maintenance of yeast were used. Preparation of medium and plates was as described (28). Transformation of yeast used either the lithium acetate method (29) or the protoplast method (30). Transformants were selected for uracil prototrophy (Ura^+).

RESULTS

Mutants of *E. coli* Initiator tRNA. Fig. 1 *Left* shows the sites of mutation described in this report; the seven mutants were named according to the base changes: G72, T35A36, T1/T35A36, T35A36/G72, T35A36/G72G73, T2·A71/T35A36/G72, and G1·C72/T35A36. Mutant tRNAs with the CAU → CUA anticodon sequence change (T35A36 mutation) have the potential to read the amber termination codon. Coupling of the T35A36 mutation to mutations that generate a U1·A72 or C1·G72 base pair at the end of the acceptor stem allow the tRNAs to act as amber suppressors in *E. coli* (16). One of these mutants contains a C1·G72 base pair unique to tyrosine tRNAs in the cytoplasm of eukaryotic cells, including yeast (Fig. 1 *Right*). Therefore, we have examined whether some

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Abbreviation: TyrRS, tyrosyl-tRNA synthetase.

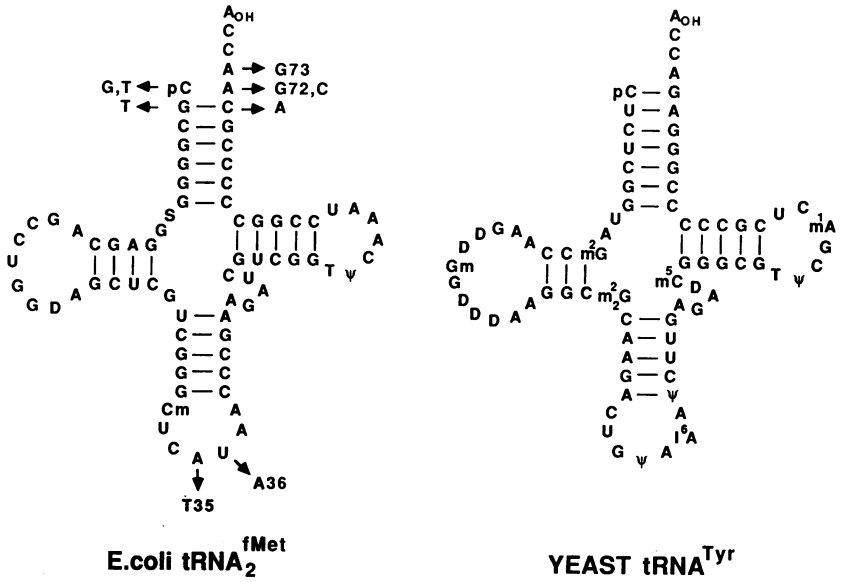

FIG. 1. Cloverleaf structures of *E. coli* initiator tRNA (*Left*) and yeast cytoplasmic tyrosine tRNA (*Right*). Arrows indicate sites of mutations studied in this report.

of these mutant tRNAs (*i*) suppress amber codons in the yeast *S. cerevisiae* and (*ii*) are aminoacylated with tyrosine.

Expression of Mutant *E. coli* Initiator tRNA in Yeast. To function in yeast, the mutant initiator tRNA genes must be expressed and the transcript must be processed to produce mature tRNA in the heterologous system. The *E. coli* $tRNA_2^{fMet}$ gene contains sequences which match the A- and B-box consensus elements (Fig. 2*A*) that are found in eukaryotic tRNA genes and that are needed for transcription by RNA polymerase III (31). The *E. coli* tRNA gene also contains a stretch of T residues required for termination of RNA polymerase III transcription, although these residues are much further downstream of the tRNA coding sequence (Fig. 2*B*) than in most eukaryotic tRNA genes. *In vitro* transcription experiments using yeast extracts (27) indicated that the *E. coli* initiator tRNA gene can be transcribed and that the transcript can be processed to yield a product of the size of mature tRNA (data not shown). Therefore, we used the *E. coli* tRNA gene without any changes to shorten the distance between the tRNA coding region and the stretch of T residues.

Northern blot analysis (32) shows that transformants containing two of the mutant initiator tRNA genes produce a transcript of the size of mature initiator tRNA (Fig. 3). This RNA is not present in yeast that was not transformed (no plasmid DNA) or transformed with vector alone (YEP420). The transcript derived from the T35A36 mutant (Fig. 1), which carries a C1–A72 mismatch at the end of the acceptor stem, is processed to yield mature tRNA. This suggests that in yeast, as in *E. coli*, an initiator tRNA with such a mismatch is recognized by the processing enzymes.

Some *E. coli* Initiator tRNA Mutants Suppress Amber Codons in Yeast. HEY301-129 cells (*ura3-1 met8-1am trp1-1am his4-580am*; see *Materials and Methods* for complete genotype) were transformed with YEP420 recombinants carrying the various mutant initiator tRNA genes with the CAU → CUA anticodon sequence change. The Ura^+ transformants were grown in liquid medium lacking uracil and screened for suppression of amino acid auxotrophy by streaking on plates lacking one of the three amino acids (Met^- or His^- or Trp^-). Fig. 4 shows that two of the mutant *E. coli* initiator tRNA genes, T35A36/G72 and T2·A71/T35A36/G72, which have a C1·G72 base pair at the end of the acceptor stem, can suppress all three amber mutations in yeast, as indicated by their growth on minimal plates lacking one of the three amino acids. The T1/T35A36 mutant, which has a U1·A72 base pair, was inactive in suppression (data not shown). Suppression requires the presence of tRNA genes on the plasmid, as no suppression was observed in HEY301-129 transformed with vector alone (YEP420). Furthermore, in plasmid segregation analysis, transformed cells that had lost the 2-μm plasmid following growth in rich medium and were phenotypically Ura^- were also phenotypically suppressor-negative as indicated by lack of growth on plates that contained uracil but lacked one of the amino acids (data not shown).

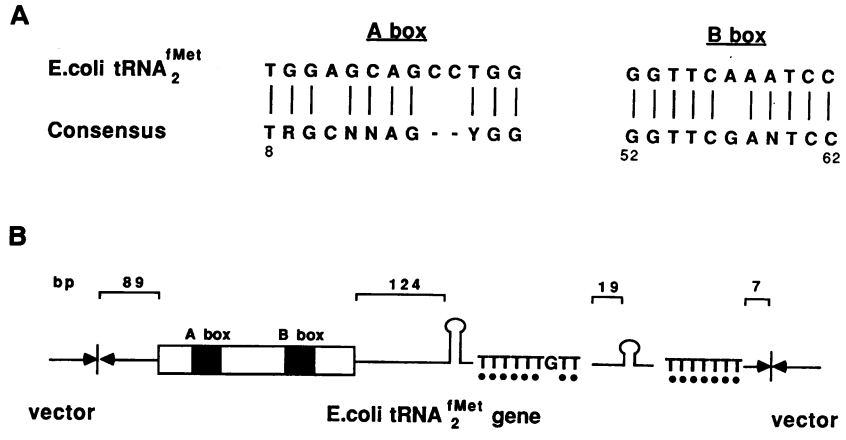

FIG. 2. (*A*) Alignment of consensus RNA polymerase III internal promoter sequences (A and B boxes) in eukaryotic tRNA genes with corresponding sequences found in *E. coli* $tRNA_2^{fMet}$ gene. (*B*) Schematic arrangement of potential recognition signals for eukaryotic RNA polymerase III in the ≈450-bp DNA fragment containing the *E. coli* $tRNA_2^{fMet}$ gene. The locations of A and B boxes are shown. Dots highlight two stretches of T residues that are potential transcription termination signals. The fragment includes 89-bp (5′) and 197-bp (3′) sequences that flank the mature tRNA coding sequence. Two hairpin structures preceding the T stretches are also indicated.

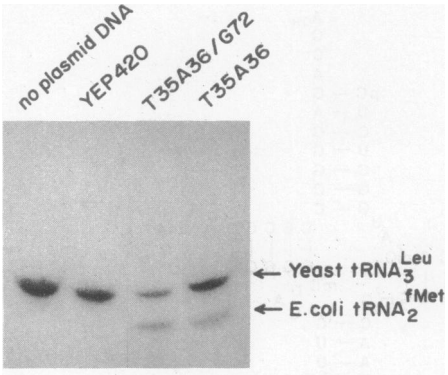

FIG. 3. Northern blot analysis of total tRNA from yeast strains untransformed or transformed with various recombinant plasmids. Liquid cultures (30 ml) of HEY301-129 transformants carrying the various mutant tRNA genes were grown to an OD_{600} of ≈1 in SD minimal medium supplemented with amino acids as required. These cultures were used to prepare total tRNAs (32) for Northern blotting and hybridization. Two oligonucleotides, each 19 bases long, were used as hybridization probes. One has sequence complementary to nucleotides 29–47 of *E. coli* $tRNA_2^{fMet}$; the other has sequence complementary to nucleotides 29–47 of yeast $tRNA_3^{Leu}$.

Aminoacylation of *E. coli* Initiator tRNA Mutants with Tyrosine by Yeast Extracts. Fig. 5 shows results of aminoacylation assays carried out on a number of mutant initiator tRNAs. The tRNAs were expressed in *E. coli* and were purified by gel electrophoresis (26). Of the mutants tested, the best substrate for yeast TyrRS is the T35A36/G72 mutant. The next best substrate is the T2·A71/T35A36/G72 mutant, which has an additional change of the C2·G71 base pair to U2·A71. The slight decrease in activity of this mutant may not be significant, since another preparation of this mutant tRNA was as active in tyrosine acceptance as the T35A36/G72 mutant. Furthermore, although yeast tyrosine tRNA contains a U2·A71 base pair, this base pair is not conserved among eukaryotic tyrosine tRNAs (24). The presence of a C1·G72 base pair appears crucial for aminoacylation with tyrosine. Mutants that contain a G1·C72 base pair (G1·C72/T35A36) or a U1·A72 base pair (T1/T35A36) are not aminoacylated to any measurable extent (Fig. 5*A*).

The T35A36/G72 mutant contains changes in the anticodon sequence and in the acceptor stem. The anticodon change alone (T35A36 mutant) is not enough to make the *E. coli* initiator tRNA a substrate for yeast TyrRS (Fig. 5*B*). On the other hand, the G72 mutant, which has a C1·G72 base pair but the wild-type anticodon, can be partly aminoacylated with tyrosine, although not as well as the T35A36/G72 mutant. This result reinforces the importance of the C1·G72 base pair for aminoacylation by yeast TyrRS.

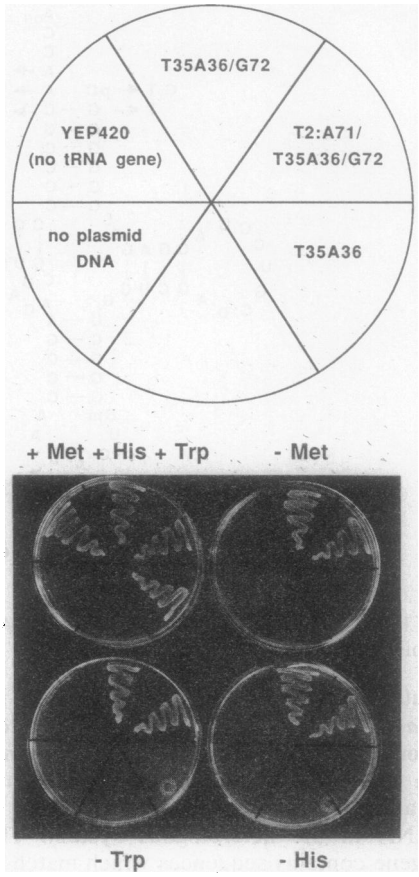

FIG. 4. Test for suppression of amber alleles in *S. cerevisiae*, using growth of HEY301-129 (*ura3-1 met8-1am, trp1-1am, his4-580am*) on selective plates lacking methionine, histidine, or tryptophan. Plates shown here were incubated for 2–4 days at 30°C, depending upon the amber allele whose suppression was being scored (25). Ura^+ transformants of HEY301-129 were picked, grown in SD medium lacking uracil, and streaked on the selective plates. None of the four plates contained uracil. Key to the identity of the plates is provided at the top.

The results also indicate a role for the "discriminator" base A73 (34) in aminoacylation of tRNA by yeast TyrRS. Introduction of an additional mutation (A73 → G73) into the T35A36/G72 mutant lowers aminoacylation of the resulting mutant tRNA (T35A36/G72G73) to near background (Fig. 5*B*). The discriminator base is also important in aminoacylation of tRNA by *E. coli* TyrRS (35).

Kinetic Parameters in Aminoacylation of tRNA by Yeast TyrRS. Table 1 compares the kinetic parameters in aminoacylation of the T35A36/G72 mutant of *E. coli* initiator

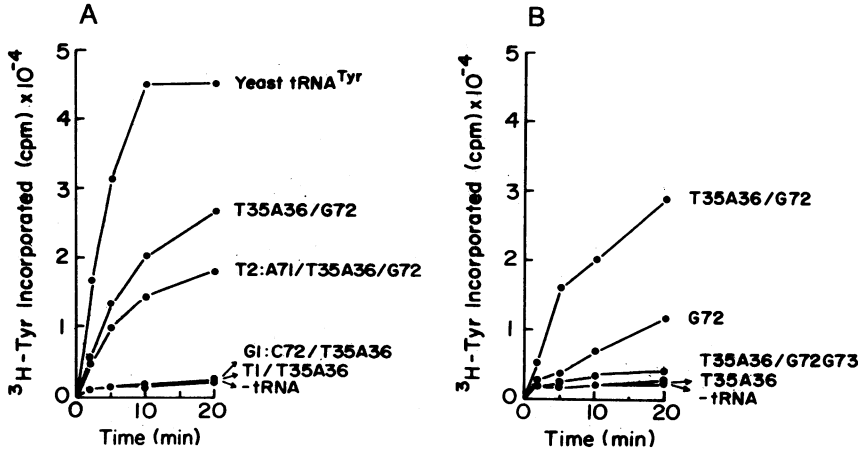

FIG. 5. Time course of aminoacylation of mutant *E. coli* initiator tRNAs with tyrosine by yeast extracts. Mutant *E. coli* initiator tRNAs genes were expressed in a strain of *E. coli* B that lacks endogenous $tRNA_2^{fMet}$. Purification of mutant tRNAs by polyacrylamide gel electrophoresis and estimation of their purity by aminoacylation with pure *E. coli* GlnRS were as described (16, 33). Aminoacylation reaction mixtures (30 μl) were incubated at 37°C and contained 20 mM imidazole/HCl (pH 7.5), 0.1 mM EDTA, 2 mM ATP, 150 mM NH_4Cl, bovine serum albumin (10 μg/ml), 4 mM $MgCl_2$, 34 μM [^{3}H]tyrosine (104 Ci/mmol; 1 Ci = 37 GBq), a saturating amount of yeast TyrRS (supplied from yeast extracts), and 2 μg of tRNA. Aliquots (6.5 μl) were taken at various times for measurement of acid-insoluble radioactivity.

Table 1. Kinetic parameters in aminoacylation of yeast $tRNA^{Tyr}$ and the T35A36 mutant *E. coli* initiator tRNA with tyrosine by yeast extracts

tRNA	Experiment 1			Experiment 2		
	K_m, μM	V_{max}*	V_{max}/K_m^{app}	K_m, μM	V_{max}*	V_{max}/K_m^{app}
$tRNA^{Tyr}$	2.86	1.67	0.584	2.7	2.5	0.926
T35A36/G72 mutant	25	0.71	0.028	28.6	0.77	0.027

The reaction was carried out as described in the legend to Fig. 5 except that limiting amounts of yeast extracts and varying amounts of tRNA (0.29–2.35 μM for yeast $tRNA^{Tyr}$ and 1.33–8 μM for T35A36/G72 mutant tRNA) were used. Incubation was at 37°C for 3 min. An aliquot (25 μl) was taken for measurement of acid-insoluble radioactivity.
*Arbitrary units.

tRNA (anticodon sequence CUA) with those of yeast $tRNA^{Tyr}$ having the anticodon sequence GψA. The V_{max}/K_m^{app} ratio of the T35A36/G72 mutant is 21–34 times lower than that of yeast tyrosine tRNA. Most of this difference is due to differences in K_m. In their analysis of an anticodon sequence mutant of yeast tyrosine tRNA in which the anticodon sequence was changed from GψA to CUA, Bare and Uhlenbeck (21) found a similar difference in V_{max}/K_m^{app} (37-fold) between the wild-type $tRNA^{Tyr}$ and mutant tRNA. Thus, the T35A36/G72 mutant of *E. coli* initiator tRNA (which has the CUA anticodon) may be as good a substrate for yeast TyrRS as the yeast amber suppressor tRNA with the same anticodon. The K_m values for tyrosine tRNA determined by Bare and Uhlenbeck (ref. 21; 50–490 nM depending on buffers and ionic conditions used) are lower than those determined here (2.7 μM). This could also be due to differences in buffer conditions used for assay.

DISCUSSION

We have shown that some mutants of *E. coli* initiator tRNA can suppress amber codons in yeast. Of the mutants tested, only those with a C1·G72 base pair, unique to and conserved in cytoplasmic tyrosine tRNAs in eukaryotes, were active in suppression. While we have not identified directly the amino acid inserted at the sites of amber mutation, it is most likely to be tyrosine. First, the amber alleles being tested are known to be suppressible by insertion of tyrosine (25, 36). Second, one of the two *E. coli* initiator tRNA mutants (T35A36/G72) active in suppression in yeast is as good a substrate for yeast TyrRS as the amber suppressor derived from yeast tyrosine tRNA (see below). Third, the T1/T35A36 mutant with a U1·A72 base pair, which is not aminoacylated with tyrosine *in vitro*, does not suppress any of the three amber alleles in yeast, although it suppresses amber codons in *E. coli*. Either this tRNA is aminoacylated very poorly in yeast or the amino acid inserted is incompatible with function of any of the three proteins (36). A third possibility, which is unlikely, is that the aminoacylated tRNA does not function in protein synthesis in yeast.

The only previous example of function of an *E. coli* tRNA in yeast cytoplasm is that of an amber suppressor derived from *E. coli* tyrosine tRNA. This tRNA is not aminoacylated by yeast TyrRS. However, by transforming yeast with two plasmids, one containing this tRNA gene and the other the *E. coli* TyrRS gene, Edwards and Schimmel (25) demonstrated that the *E. coli* tRNA could suppress amber codons in yeast. When produced in very large amounts, the *E. coli* tRNA alone was shown to suppress one of the three amber alleles (37). Under these conditions, the *E. coli* tRNA is aminoacylated almost exclusively with leucine. The need for substantial overproduction of this tRNA and the very weak suppression indicate that aminoacylation of *E. coli* tyrosine tRNA with leucine is poor *in vivo*. Thus, when the U3·A70 base pair of the *E. coli* tyrosine tRNA was replaced with G3·U70, an identity determinant for alanine tRNAs, the mutant *E. coli* tRNA inserted alanine instead of leucine (38).

Aminoacylation assays on mutant *E. coli* initiator tRNAs with yeast extracts have shown that the presence of a C1·G72 base pair is crucial for tyrosine acceptance. Thus, this base pair is a strong identity determinant for tyrosine tRNA in the cytoplasm of yeast. Since the C1·G72 base pair is unique to and conserved in tyrosine tRNAs of all eukaryotic cytoplasm and of archaebacteria (24), it is likely to be an identity determinant for all of them. The absolute requirement of the C1·G72 base pair for yeast TyrRS recognition could explain why *E. coli* tyrosine tRNA with a G1·C72 base pair is not aminoacylated with tyrosine in yeast unless *E. coli* TyrRS is also produced (25).

The finding that the C1·G72 base pair cannot be replaced with G1·C72 or U1·A72 for yeast TyrRS recognition suggests that the enzyme contacts this base pair directly. If so, this contact is likely to be in the major groove of the base pair, since C·G and G·C base pairs are thought to possess basically similar functional groups in the minor groove at more or less identical positions (39). The major groove of an RNA helix is narrow and deep, in contrast to the DNA B helix, and hence it is thought that proteins recognize sequences in RNA double-helical regions primarily through the shallow, minor-groove side. The crystal structure of a GlnRS–$tRNA^{Gln}$ complex provided an example of recognition through the minor-groove side of base pairs 2·71 and 3·70 of $tRNA^{Gln}$ by GlnRS (13). Consistent with the idea that C·G and G·C base pairs display similar functional groups in the minor groove, mutant tRNAs that have C3·G70 or G3·C70 base pairs are good substrates for GlnRS whereas those with A3·U70 or U3·A70 are poor substrates (14, 40). At the end of RNA helices, however, the major groove of base pairs is accessible for interaction with proteins, as seen in the crystal structure of yeast AspRS–$tRNA^{Asp}$ complex (20).

Aminoacyl-tRNA synthetases can be broadly classified into two structural types (41). Those of class I contain a characteristic Rossmann fold found in the dinucleotide-binding domain of dehydrogenases and kinases, whereas those of class II lack such a domain and contain instead a nucleotide-binding domain built around a central seven-stranded antiparallel β-sheet. There is also an excellent functional correlation between this classification based on structure and conserved sequences and the choice by the enzyme of 2′- or 3′-OH group for aminoacylation. Class I enzymes attach the amino acid to the 2′-OH group whereas class II enzymes attach it to the 3′-OH group. Yet another apparent correlation has recently emerged from crystal structures of two aminoacyl-tRNA synthetase–tRNA complexes. GlnRS, belonging to class I, interacts with the acceptor stem of tRNA from the minor-groove side (13) whereas AspRS, belonging to class II, interacts with the acceptor stem from the major-groove side. These observations have led to the notion that the way in which aminoacyl-tRNA synthetase interacts with tRNA can be generalized as belonging to two categories depending upon whether it is a class I or a class II enzyme (20). With yeast TyrRS, as discussed above, it is likely that the enzyme interacts with the 1·72 base pair in the major groove rather than in the minor groove. However, from

its preference of aminoacylation at 2'-OH over 3'-OH (42, 43), it would, like *E. coli* TyrRS (44), seem to belong to class I. It should be interesting to determine the sequence of yeast TyrRS to see whether it has sequence and structural motifs typical of class I or class II aminoacyl-tRNA synthetases.

Besides the C1·G72 base pair, the discriminator base A73 could also play an important role in aminoacylation. Changing A73 to G73 makes the mutant tRNA a poorer substrate (Fig. 5*B*, compare data on T35A36/G72G73 and T35A36/G73) for yeast TyrRS. Whether this is due to loss of an important contact or due to negative interactions is not known. The occurrence of A73 in many other tRNAs does not preclude an important role for it in aminoacylation by yeast TyrRS. In the crystal structure of yeast AspRS–$tRNA^{Asp}$ complex, the enzyme is seen to make close contact with the discriminator base G73, another nucleotide common to many yeast tRNAs (20). In accordance with this, mutational analysis shows that a change of G73 to any one of the other three nucleotides lowers V_{max}/K_m by a factor of 36–200, depending on the nucleotide change (18).

Of the initiator tRNA mutants analyzed, the best substrate for yeast TyrRS is the T35A36/G72 mutant. The V_{max}/K_m^{app} for aminoacylation of this mutant by TyrRS is between 21–34 times lower than that of wild-type yeast tyrosine tRNA. This difference is similar to that found by Bare and Uhlenbeck (21) between wild-type yeast tyrosine tRNA and the amber suppressor with the CUA anticodon. Thus, the T35A36/G72 amber suppressor derived from *E. coli* initiator tRNA is as good a substrate for yeast TyrRS as the amber suppressor derived from yeast tyrosine tRNA. These results indicate that the anticodon nucleotides identified by Bare and Uhlenbeck (21) and the C1·G72 base pair and discriminator base identified in this work may comprise the critical identity determinants for tyrosine tRNAs in yeast. Are these few nucleotides sufficient for yeast TyrRS to discriminate tyrosine tRNA from all other tRNAs in yeast or are other sequence elements, such as the C3·G70 base pair common to both yeast tyrosine tRNA and *E. coli* initiator tRNA (Fig. 2), also important? This could be examined by mutations of the C3·G70 base pair and by transplanting the C1·G72 base pair and A73 into other sequence backgrounds, as was done to study other aminoacyl-tRNA synthetase–tRNA recognitions (5–7).

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