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A Bacterial Amber Suppressor in *Saccharomyces cerevisiae* Is Selectively Recognized by a Bacterial Aminoacyl-tRNA Synthetase

HELEN EDWARDS AND PAUL SCHIMMEL*

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Little is known about the conservation of determinants for the identities of tRNAs between organisms. We showed previously that *Escherichia coli* tyrosine tRNA synthetase can charge the *Saccharomyces cerevisiae* mitochondrial tyrosine tRNA in vivo, even though there are substantial sequence differences between the yeast mitochondrial and bacterial tRNAs. The *S. cerevisiae* cytoplasmic tyrosine tRNA differs in sequence from both its yeast mitochondrial and *E. coli* counterparts. To test whether the yeast cytoplasmic tyrosyl-tRNA synthetase recognizes the *E. coli* tRNA, we expressed various amounts of an *E. coli* tyrosine tRNA amber suppressor in *S. cerevisiae*. The bacterial tRNA did not suppress any of three yeast amber alleles, suggesting that the yeast enzymes retain high specificity in vivo for their homologous tRNAs. Moreover, the nucleotides in the sequence of the *E. coli* suppressor that are not shared with the yeast cytoplasmic tyrosine tRNA do not create determinants which are efficiently recognized by other yeast charging enzymes. Therefore, at least some of the determinants that influence in vivo recognition of the tyrosine tRNA are specific to the cell compartment and organism. In contrast, expression of the cognate bacterial tyrosyl-tRNA synthetase together with the bacterial suppressor tRNA led to suppression of all three amber alleles. The bacterial enzyme recognized its substrate in vivo, even when the amount of bacterial tRNA was less than about 0.05% of that of the total cytoplasmic tRNA.

In vivo and in vitro studies have identified specific nucleotides as the major determinants for recognition of several tRNAs by their cognate aminoacyl-tRNA synthetases (31, 38). In vivo recognition studies have taken advantage of amber suppression in *Escherichia coli* as an assay for the function of tRNA sequence variants. In this approach, the role of the anticodon in recognition cannot be assessed because of the obligatory CUA anticodon. However, 11 of 20 amber suppressors that were tested in *E. coli* retain the correct amino acid specificity and thus are amenable to this method of study (31). As demonstrated by amino acid sequence analysis of a suppressed protein, properly chosen nucleotide substitutions in a tRNA amber suppressor can specify attachment of a different amino acid and thereby change the identity of the tRNA (17, 30, 32).

In early in vitro studies, the interaction between an aminoacyl-tRNA synthetase and tRNAs from different organisms was analyzed. Heterologous tRNAs supplied natural sequence variants for studies of recognition (35). More recent in vitro studies with synthetic tRNA variants and purified aminoacyl-tRNA synthetases have established systems which mimic the specificity of aminoacylation observed in vivo and thus identify nucleotides essential for recognition. Here also, the specificity of aminoacylation of a tRNA can be altered by introduction of identity elements from another tRNA (36, 39).

For many tRNAs, it is not known whether the identity elements are conserved in evolution. In a recent study, we demonstrated that *E. coli* tyrosyl-tRNA synthetase can substitute for the function of the *Saccharomyces cerevisiae* mitochondrial tyrosyl-tRNA synthetase in vivo, even though the yeast mitochondrial tyrosine tRNA substrate differs in sequence from the *E. coli* tRNA (13). This implies that determinants for the identity of tyrosine tRNA have been conserved between yeast mitochondria and *E. coli*. These two species of tRNA are representatives of a less common type of tRNA (type II), which, among other features, have large variable loops, ranging from 13 to 21 nucleotides (13 or 14 nucleotides in these cases).

In contrast, the substrate of the yeast cytoplasmic tyrosyl-tRNA synthetase is a member of the more common type I class of tRNA. These tRNAs generally have a short variable loop of four or five nucleotides. Figure 1 compares the sequences and cloverleaf structures for *E. coli* and yeast cytoplasmic tRNAs (42). Although these tRNAs are in different structural classes, they do have some similarities; in addition to 23 nucleotides that are invariant or semi-invariant in all tRNAs, the *E. coli* and yeast cytoplasmic tRNAs have 19 nucleotides in common. Only 8 of these 19 nucleotides are also common to the mitochondrial tyrosine tRNA (42), which can be charged by the *E. coli* enzyme (13). Previous experiments with crude synthetase preparations have shown that in vitro there is little, if any, aminoacylation with tyrosine of *E. coli* tyrosine tRNA by a crude preparation of yeast aminoacyl-tRNA synthetases (4, 9, 11). The question of whether the *E. coli* tyrosine tRNA could be aminoacylated by other yeast enzymes was not addressed.

We set up an in vivo system to address the possibility of interactions between the *E. coli* tRNA and the yeast cytoplasmic enzyme. This in vivo system tests for recognition in the presence of a full complement of aminoacyl-tRNA synthetases and tRNAs and is therefore sensitive to competition effects and their contribution to identity. We tested for suppression of nuclear encoded amber mutations in *S. cerevisiae* by an *E. coli* tyrosine tRNA amber suppressor. The tyrosine suppressor tRNA is one of those tRNAs (see above) that, in *E. coli*, retains its specificity in vivo despite its altered anticodon (*GUA to CUA). Also, although the efficiency of aminoacylation in vitro is reduced, the tRNA is a strong suppressor in *E. coli* (18). In vitro, the yeast

* Corresponding author.

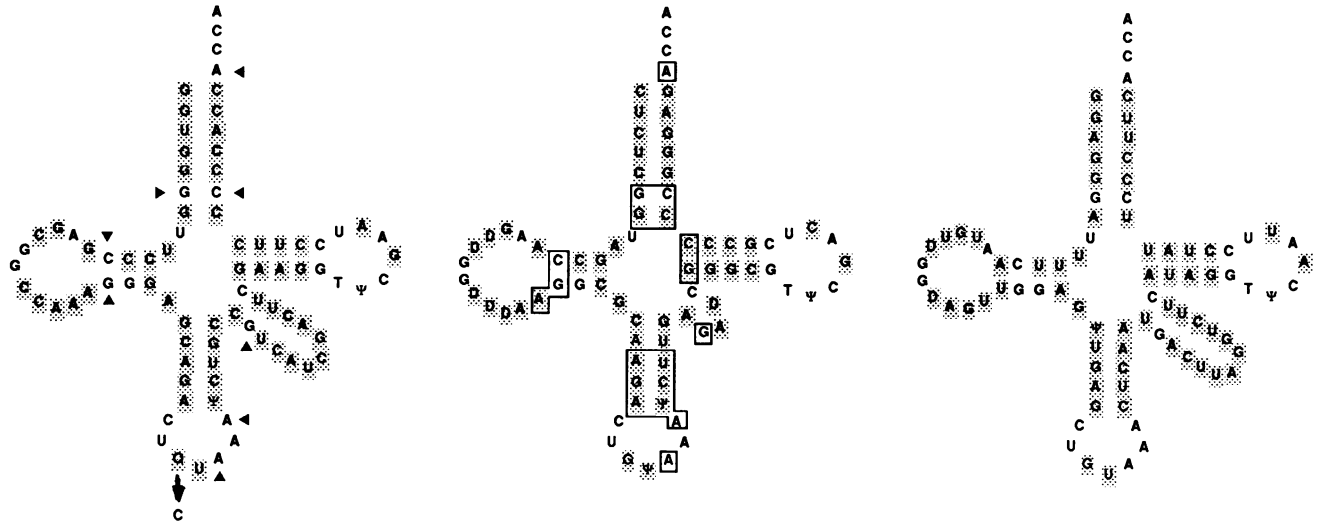

FIG. 1. Comparison of tyrosine tRNA structures. The *E. coli*, *S. cerevisiae* cytoplasmic, and the *S. cerevisiae* mitochondrial tyrosine tRNAs are shown in schematic cloverleaf form. Nucleotides that are not identical in all three tRNAs are shaded. The arrowheads point to eight residues in the *E. coli* tRNA that are identical in all three tRNAs but are not universally conserved among all tRNAs. The boxed nucleotides on the yeast cytoplasmic tRNA are bases that are common to this tRNA and to the *E. coli* tRNA, but are not universally conserved. Note that the tRNAs differ in the number of nucleotides in the D-loop and the variable loop, and in the number of base pairs in the D-stem.

tyrosine tRNA amber suppressor can be charged by its cognate synthetase, although the efficiency of aminoacylation is reduced (3).

Constructions to express different amounts of the *E. coli* tyrosine tRNA suppressor in *S. cerevisiae* were designed. These plasmids were introduced into a tester strain which has three suppressible amber alleles. All three amber codons can be suppressed either by insertion of tyrosine, or, in some cases, by insertion of one of several other amino acids. For example, the *met8-1* allele is known to be suppressed by insertion of tyrosine, leucine, phenylalanine, or tryptophan (22–24, 28), whereas the *trp1-1* allele is known to be suppressed by tyrosine, leucine, serine, or glutamine (7, 23, 24, 45). There may be other amino acids, as yet untested, which also suppress these amber alleles.

Thus, our system is sensitive to possible aminoacylation of the *E. coli* tyrosine tRNA by noncognate yeast synthetases, whether the *E. coli* tyrosine suppressor tRNA has determinants for recognition by multiple synthetases or has a relatively narrow specificity for a single amino acid. Moreover, because amber suppression in *E. coli* has been observed even when the suppressor tRNA is inactive for aminoacylation in vitro (33), this in vivo assay potentially is also sensitive to weak interactions between synthetases and a tester tRNA that lead to aminoacylation.

MATERIALS AND METHODS

Strains and genetic methods. Bacterial strain DH1 was used for plasmid propagation and isolation. Strains JM101 and TG1 were used for propagation and isolation of bacteriophage DNA. Strain XAC-I [F′ *lacI373 lacZu118(Am) proB*$^+$/F$^-$ Δ(*lac proB*) *χ111 nalA rif8 argE*(Am) *ara*] was used to screen plasmids for insertion of the amber suppressor gene. (XAC-I was a kind gift from Ya-Ming Hou, Massachusetts Institute of Technology.)

The following yeast strains were used in these studies: W303-1B (α *trp1-1 leu2-3,112 ura3-1 ade2-1 his3-11,15 can 1-100*), GRF11 (**a** *met8-1 trp1-1 lys1-1 arg4-17 his5 ade1*), 2425/3 (α *met8-1 his4-580*), L833 (α *met8-1 trp1-1 lys1-1 arg4-17 leu2-2 ade1*), L3433 (α *aro7-1 his4*), HEY300-103 (**a** *met8-1 trp1-1 leu2-3,112 ura3-1 ade1 lys1-1 can1-100*), HEY301-129 (**a** *met8-1 trp1-1 his4-580 leu2-3,112 ura3-1 ade1 can1-100*), HEY303-66 (**a** *met8-1 aro7-1 leu2-3,112 ura3-1 ade1 lys1-1 can1-100*), and HEY205-2B (**a** *leu2-3,112 ura3-1 his3 msy1-1*). (Strains GRF11, 2425/3, L833, and L3433 were gifts from G. Fink, Massachusetts Institute of Technology, Whitehead Institute). Yeast strains HEY300-103, HEY301-129, and HEY303-66 were obtained by random spore analysis of W303-1B and GRF11, 2425/3 and HEY300-103, and L3433 and HEY300-103, respectively. Random spore analysis was performed as described by Sherman et al. (41) with the following modifications: glusulase digestion was carried out for 10 min at room temperature, glass beads were added to the digestion immediately to make a slurry, and asci were disrupted by vortex mixing for 1 min before plating dilutions on SD (see below) containing canavanine and required amino acids. The *ade1* genotype of HEY300-103 was deduced from complementation tests in which it failed to complement the *ade1* allele of L833 but did complement the *ade2-1* defect of W303-1B.

Standard techniques for growth and maintenance of *S. cerevisiae* strains were used (41). Yeast transformation was performed by the method of Hinnen et al. (16). Synthetic minimal media and YEP medium (41) were supplemented with 2% glucose to give SD and YPD, respectively.

Plasmid constructions. A *Hin*dIII-*Eco*RI fragment from pRD3 (a kind gift from U. L. RajBhandary, Massachusetts Institute of Technology [12]), which carries the suIII *E. coli* amber suppressor tyrosine tRNA, was cloned into the *Hin*dIII and *Eco*RI sites of M13mp18. This construction was used as a template for oligonucleotide-directed mutagenesis with the 25-mer 5′-CCCACCACCATTTTTTTCAAAAGTC-

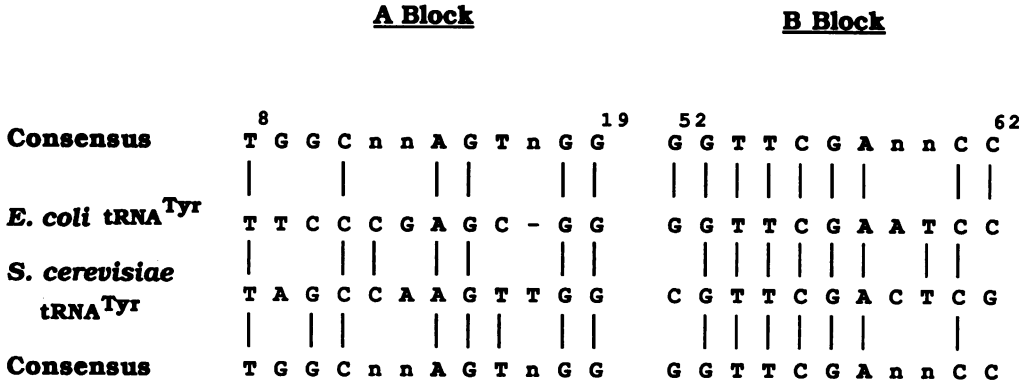

FIG. 2. Internal promoter sequences of tRNAs. The yeast tyrosine tRNA and consensus eucaryotic internal tRNA promoter sequences (A and B boxes) are shown. The corresponding internal region of the *E. coli* tyrosine tRNA is shown for comparison. Standard tRNA numbering is used to indicate the nucleotide positions of the two sequence blocks. Dashes indicate optional nucleotides; n indicates that the position is not conserved. Vertical lines indicate a sequence match.

3′ to introduce a sequence of seven consecutive T residues. (The three nucleotide changes are underlined.) Protocols and reagents for mutagenesis were from Amersham Corp. The mutant was identified by sequencing (5, 37), using universal primer from New England BioLabs, Inc.

The *Hin*dIII-*Eco*RI fragment encoding the tRNA, with the appropriately altered 3′-flanking region, was purified, and the ends were made flush with Klenow enzyme. *Sal*I linkers were added, and then the fragment was cloned into the *Sal*I site of vector YEp21 (6). Ligation mixes were used to transform *E. coli* XAC-I, and ampicillin-resistant colonies were selected. Colonies were screened for the presence of insert (encoding the suppressor tRNA) by phenotypic suppression of the *lacZ* and *argE* amber alleles on minimal medium containing 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

The number of inserts and their orientations in the constructions isolated, pHET100am and pHET120am, were determined by restriction analysis and DNA sequencing. Plasmids pHET100am and pHET120am were recovered from *S. cerevisiae* in which suppression was observed (10). The *Pvu*II-*Eco*RV fragments from these vectors, which contain the gene for the tRNA amber suppressor, were isolated and cloned into the *Sma*I site of M13mp18. A 19-mer complementary to a region near the *Sal*I site of the insert was used as a primer for sequencing.

Plasmid pHET400am is a derivative of pHET100am. An *Eag*I-*Eco*RI fragment, which carries the gene for the tRNA, was isolated from pHET100am. A 1-μg portion of this fragment and 200 ng of *Sal*I-cut YCp402 (25) were used to transform the Leu$^-$ yeast strain HEY205-2B. Leu$^+$ transformants were selected, and a plasmid was isolated which had incorporated the tRNA gene by recombination (25).

Plasmid pHEF300Δ1 contains the same insert as plasmid pHEF200Δ1 (13). Plasmid pHEF200Δ1 carries a version of the *E. coli* tyrosyl-tRNA synthetase gene which is fused to the promoter of the yeast mitochondrial tyrosyl-tRNA synthetase. In this construction, the first 8 codons after the initiator methionine of the bacterial enzyme are replaced by the corresponding codons 52 to 59 from the yeast mitochondrial enzyme. The *Sal*I fragment encoding this gene was cloned into the *Sal*I site of YCp50 (21), thus disrupting the tetracycline resistance gene. Ampicillin-resistant colonies (that were also tetracycline resistant) were recovered, and the presence and orientation of the insert were determined by restriction digestion.

Northern (RNA) analysis. Yeast RNA was prepared as described previously (8), after growth on selective media. RNA was separated on 10% polyacrylamide–7 M urea gels and transferred electrophoretically to nylon-66 filters (Schleicher & Schuell, Inc.) as described elsewhere (M. Francis and U. L. RajBhandary, manuscript in preparation). The gel was equilibrated in transfer buffer (10 mM Tris base, 5 mM sodium acetate, 0.5 mM EDTA [pH 7.8]) for 30 min, and transfer was performed at 10 V for 30 min and at 40 V for another 2 h at 4°C. Filters were processed as specified by the manufacturer for hybridization with oligonucleotide probes for yeast tRNA (5′-CTCCCGGGGGCGAG-3′) and for *E. coli* tRNA (5′-GGTGGGGGAAGGAT-3′), except that hybridization solutions contained 5× Denhardt solution and hybridizations were done at room temperature. Specific activities of the oligonucleotides, following labeling with [γ-^{32}P]ATP, were determined by scintillation counting of oligonucleotides that were separated from unincorporated counts by polyethyleneimine cellulose chromatography (29). The *Sal*I fragment encoding the tRNA from pHET120am was used as a probe after nick translation (26). Hybridization with this probe (4.0×10^6 Cerenkov counts) was performed as follows. The prehybridization and hybridization solution contained 50% formamide, 5× SSPE, 5× Denhardt solution, and 0.5% sodium dodecyl sulfate; hybridization at 37°C was performed overnight, followed by two 20-min washes in 2× SSPE–0.1% sodium dodecyl sulfate at 37°C. *E. coli* tyrosine tRNA (Boehringer Mannheim Biochemicals) was used as a standard.

RESULTS

Strategy for expression of *E. coli* tRNA in *S. cerevisiae*. Eucaryotic tRNAs are transcribed by RNA polymerase III (pol III). In several systems, the pol III promoter elements are located internal to the gene in two sequence blocks called the A box (nucleotides 8 to 19) and the B box (nucleotides 52 to 62) (15). In *S. cerevisiae* these internal regions may also be major transcriptional control regions (1).

The regions of the *E. coli* tyrosine tRNA corresponding to the A and B boxes are similar to their yeast tyrosine tRNA counterparts and to a consensus sequence drawn from 80 eucaryotic tRNAs (Fig. 2) (12, 42). The *E. coli* A box sequence has six of nine matches to the eucaryotic consensus A box, and all six matches are shared with the yeast cytoplasmic tRNA. (The yeast sequence, however, has an

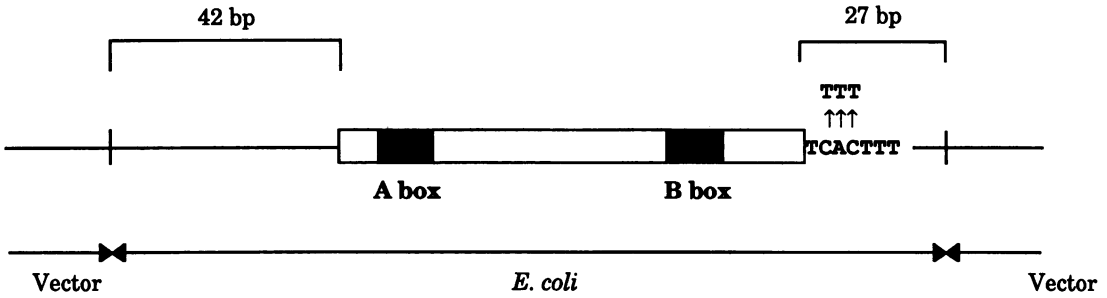

FIG. 3. Structure of the *E. coli* tyrosine amber suppressor tRNA gene expressed in *S. cerevisiae*. The region of the *E. coli* tyrosine amber suppressor tRNA gene which encodes the mature tyrosine tRNA is shown as a box. The location of the A and B boxes (sequences similar to eucaryotic internal promoter regions) are shown (■). Three nucleotides at the 3′ end of the tyrosine tRNA gene from *E. coli* were changed to T residues as indicated. The construction retains 42 bp of 5′-flanking sequence and 27 bp of 3′-flanking sequence from *E. coli*. In each vector used in this study, the tRNA gene is cloned into the unique *Sal*I site with *Sal*I linkers.

additional two nucleotides that match the consensus. Also, an optional nucleotide, which is absent in the *E. coli* tRNA, is present in yeast tyrosine tRNA.) The sequence of the *E. coli* tyrosine tRNA from nucleotides 52 to 62 is a perfect match to the eucaryotic consensus B box sequence. Thus, to express the *E. coli* suIII amber suppressor in *S. cerevisiae*, no changes were made internal to the tRNA gene because the sequences are similar to the yeast tyrosine tRNA gene sequence and to the consensus sequence and because such changes would alter the sequence of the mature tRNA.

5′-Flanking sequences are known to modulate tRNA transcription in *S. cerevisiae* (27, 34, 40). However, because a wide variety of sequences in this region are compatible with transcription, no changes in the existing 5′-flanking sequence were made for the applications described here.

Transcription by pol III has been shown to terminate in stretches of T residues that are located on the 3′ side of the mature tRNA. A deletion analysis of the yeast *SUP4*(o) tyrosine tRNA showed that removal of T residues at the 3′ end of the gene led to readthrough transcripts that were not efficiently processed, so that the in vivo strength of suppression was reduced. A deletion mutant retaining seven T residues supported efficient termination and had a strength of suppression comparable to that of the wild-type gene (2). Therefore, to ensure proper 3′ end maturation of the *E. coli* tRNA in *S. cerevisiae*, the sequence at the 3′-CCA terminus of the gene was altered by oligonucleotide-directed mutagenesis from CCATCACTTTC to CCATTTTTTTC. This alteration gives rise to a string of seven T residues that may act as a pol III terminator in *S. cerevisiae*. The final construction retains 42 base pairs (bp) of *E. coli* DNA on the 5′ side of the sequence of the mature tRNA and 27 bp of *E. coli* DNA (with the changes mentioned above) on the 3′ side (Fig. 3).

A fragment carrying this tRNA gene was cloned into the *Sal*I site of the multicopy vector YEp21 (Fig. 4A). We obtained one isolate with a single insert (pHET100am) and another isolate with two tRNA gene inserts (pHET120am). Because high-copy expression of some suppressors has been shown to be toxic relative to low-copy vectors (28), we also cloned the gene into the *Sal*I site of the centromere plasmid YCp402. The resulting construction (pHET400am) contains a single insertion of the tRNA gene (Fig. 4B). In each vector, the 5′ end of the tRNA is downstream from the *LEU2* gene. Downstream from all of the tRNA gene inserts, the constructions have the same 1,400 bp of DNA from pBR322.

***E. coli* tRNA gene constructions direct the formation of mature tRNA in *S. cerevisiae*.** To determine whether the *E. coli* tRNA was produced in *S. cerevisiae*, total cellular RNA was obtained from *S. cerevisiae* transformed with one of the plasmids described above (pHET100am, pHET120am, or pHET400am) or with the parent vectors (YEp21 or YCp402) and was then subjected to Northern analysis. RNA was separated on a denaturing 10% polyacrylamide gel and transferred electrophoretically to nylon membranes. Filters

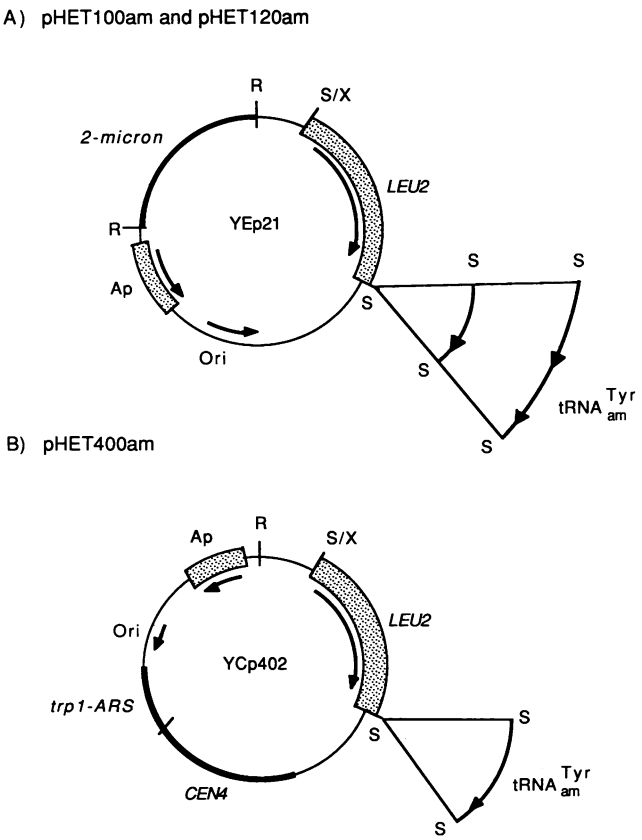

FIG. 4. Plasmids pHET100am, pHET120am, and pHET400am. The *E. coli* tyrosine amber suppressor tRNA construct shown in Fig. 3 was cloned into the *Sal*I site of YEp21 to make plasmids pHET100am and pHET120am, which contain one and two tRNA gene inserts, respectively (A). The suppressor tRNA construct was transferred into centromere (*CEN4*) plasmid YCp402 by recombination, as described in Materials and Methods, to make plasmid pHET400am (B). Restriction sites: R, *Eco*RI; S, *Sal*I; S/X, *Sal*I/*Xho*I.

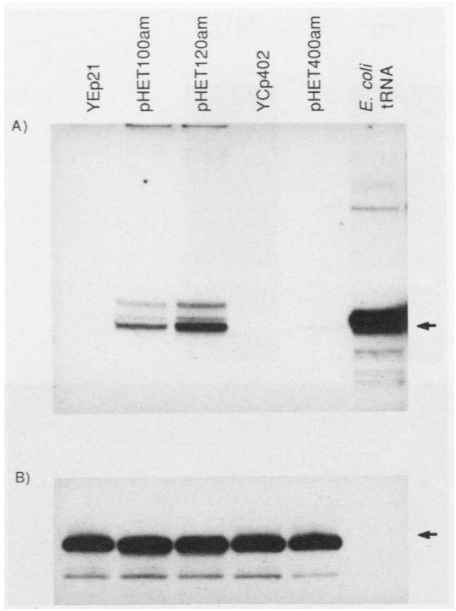

FIG. 5. Northern blot analysis of total RNA from HEY301-129 transformants. Total RNA was isolated from HEY301-129 transformants which carried plasmid YEp21, pHET100am, pHET120am, YCp402, or pHET400am. (YEp21 is the parent vector of pHET100am and pHET120am, whereas YCp402 is the parent vector of pHET400am.) The plasmid carried by each HEY301-129 transformant is shown above the corresponding lane of the gels. Pure *E. coli* tyrosine tRNA was loaded in the last lanes. Total RNA (15 μg per lane) was fractionated on 7 M urea–10% polyacrylamide gels. The RNA was transferred electrophoretically to nylon filters and treated with ^{32}P-labeled probes. (A) Northern blot probed with the *Sal*I fragment which codes for the *E. coli* tyrosine suppressor tRNA (prepared by nick translation). The origin of the two bands above the *E. coli* tRNA has not been determined. These bands are specific to the *E. coli* tRNA gene and are likely to be incompletely processed transcripts. (B) Duplicate Northern blot probed with an end-labeled 14-mer complementary to the yeast cytoplasmic tyrosine tRNA. (The lower band may be a breakdown product of the highly expressed endogenous tRNA.)

were probed with a ^{32}P-labeled *Sal*I fragment which encoded the tRNA gene. A band comigrating with pure *E. coli* tyrosine tRNA (Boehringer Mannheim) was detected in extracts of cells which carry tRNA-encoding plasmids (Fig. 5A, lanes 2, 3, and 5), but not in extracts of cells which carry vector alone (lanes 1 and 4). The results show that in *S. cerevisiae*, the gene for the *E. coli* amber suppressor is transcribed, the RNA is processed, and the resulting tRNA is comparable in size to the tyrosine tRNA that is produced in *E. coli*. Other data established that this tRNA is biologically active in *S. cerevisiae* (see below).

***E. coli* amber suppressor tyrosine tRNA produced from plasmids pHET100am, pHET120am, or pHET400am is not recognized efficiently by yeast synthetases.** To test for function of the *E. coli* tRNA in *S. cerevisiae*, we introduced our tRNA constructions by transformation into the tester strain HEY301-129. This strain carries the three suppressible amber alleles *met8-1*, *trp1-1*, and *his4-580* and the *leu2-3,112* and *ura3-1* alleles for plasmid selection. Leu$^+$ transformants were selected, patched to YPD plates (rich medium), and replica plated to synthetic minimal medium to score each marker. All pHET100am, pHET120am, and pHET400am transformants were Met$^-$ Trp$^-$ His$^-$, indicating no suppression. Thus, we could not detect aminoacylation of the *E. coli* suppressor tRNA by any of the yeast cytoplasmic aminoacyl-tRNA synthetases under these conditions.

Quantitation of *E. coli* suppressor tRNA produced in *S. cerevisiae*. The absolute level of the suppressor tRNA may be too low to achieve suppression. To determine the amount of tRNA produced relative to an endogenous yeast tRNA, we synthesized a 14-base oligonucleotide probe complementary to nucleotides 59 to 72 of the yeast cytoplasmic tyrosine tRNA. This oligonucleotide was end labeled with ^{32}P and used to probe a Northern blot of total RNA from *S. cerevisiae* carrying each of the *E. coli* tRNA plasmids or parent vectors (Fig. 5B). A second 14-nucleotide probe complementary to nucleotides 59 to 72 of the *E. coli* tRNA was also made. This oligonucleotide was also end labeled with ^{32}P and was used to probe a second set of Northern blots (data not shown). The oligonucleotides hybridized specifically with their respective tRNAs.

To compare the level of *E. coli* tyrosine tRNA with that of yeast tyrosine tRNA, we loaded 15 μg of RNA in each lane and compared lanes from the same filter. RNA transfer was consistent across the filter. After visualization by autoradiography (data not shown), bands were excised from the filters and analyzed by scintillation counting. For lanes probed with the *E. coli* tRNA oligonucleotide, we subtracted background counts of the lane with RNA of cells carrying the parent vector, YEp21, from the counts of the lanes with RNA of cells that harbored pHET100am or pHET120am. Because all lanes contained yeast tRNA, we averaged the counts from several lanes to obtain a value for endogenous yeast tyrosine tRNA.

By comparing the resulting values, we determined that there is 38-fold more yeast tyrosine tRNA than *E. coli* suppressor tRNA in cells which carry pHET100am. Cells transformed with pHET120am, which contains two tRNA inserts, have 21-fold more yeast tyrosine tRNA than *E. coli* suppressor tyrosine tRNA. Because *S. cerevisiae* cells have eight chromosomal copies of the tyrosine tRNA gene, the average yeast tyrosine tRNA gene produces only 2.5-fold more tRNA than does pHET120am. This level of expression is probably comparable to that of a functional yeast tyrosine tRNA suppressor, assuming that the endogenous suppressors are present at levels close to an average gene dose. Thus, were the *E. coli* suppressor functional in translation and efficiently aminoacylated by the yeast enzyme, we would expect to observe suppression.

We estimate the limit of detection for the tRNA signal to be about fivefold lower than the signal of cells which carry pHET120am. This limit corresponds to a level of suppressor tRNA about 100-fold lower than the level of total yeast tyrosine tRNA. The tRNA produced from the low-copy vector pHET400am must be at least 100-fold less abundant than yeast tyrosine tRNA, because the signal from the *E. coli* tRNA, as determined by scintillation counting, was not measurable above background. Collectively, therefore, this set of vectors directs synthesis of an *E. coli* amber suppressor tyrosine tRNA in *S. cerevisiae* to levels ranging from below 1% to about 5% of the level of endogenous yeast tyrosine tRNA.

***E. coli* tyrosyl-tRNA synthetase can efficiently recognize the *E. coli* amber suppressor tyrosine tRNA in *S. cerevisiae*.** To determine whether the *E. coli* suppressor can be aminoacylated in *S. cerevisiae* and function efficiently with the yeast translational apparatus, we cotransformed the tester strain with plasmids encoding the *E. coli* tyrosyl-tRNA synthetase and the *E. coli* suppressor tRNA. In contrast to the situation in the absence of homologous enzyme, we observed sup-

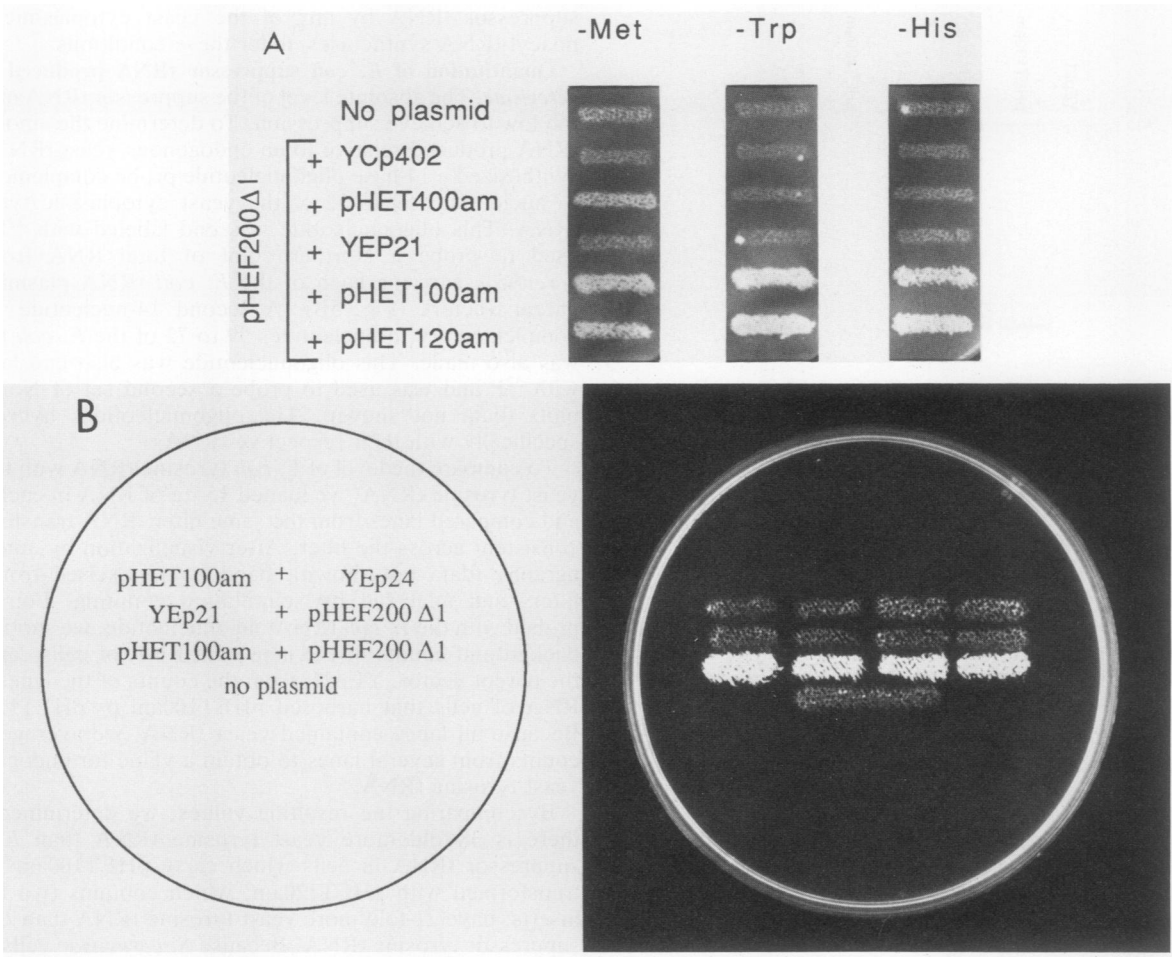

FIG. 6. Suppression of amber alleles in *S. cerevisiae* by *E. coli* suppressor tRNA and dependence on coexpression of *E. coli* tyrosyl-tRNA synthetase. Leu^+ Ura^+ cotransformants of HEY301-129 [relevant genotype *leu2-3,112 ura3-1 met8-1*(Am) *trp1-1*(Am) *his4-580*(Am)] were selected and streaked on selective minimal medium. Single colonies were patched to YPD plates and replica plated to minimal medium supplemented with the appropriate amino acids to score each marker and to score for suppression. (A) Suppression phenotypes for three amber alleles. Suppression is indicated by growth in the absence of methionine (−Met), tryptophan (−Trp), or histidine (−His). The untransformed parent (no plasmid) is shown at the top of each plate. The transformants each contain the *E. coli* tyrosyl-tRNA synthetase on a plasmid (pHEF200Δ1), plus a tRNA-encoding plasmid or parent vector. (YCp402 is the parent vector of pHET400am, whereas YEp21 is the parent vector of pHET100am and pHET120am.) (B) Dependence of suppression of the yeast *met8-1* amber allele on *E. coli* suppressor tRNA (from pHET100am) and *E. coli* tyrosyl-tRNA synthetase. The plasmids present in each patch are indicated in the map (left). Plasmid pHET100am (derived from YEp21) codes for the *E. coli* suppressor tyrosine tRNA, whereas pHEF200Δ1 (derived from YEp24) essentially codes for the *E. coli* tyrosyl-tRNA synthetase. Suppression of the *met8-1* allele is indicated by growth in the absence of methionine (plate shown).

pression. For these experiments, the *E. coli* tyrosyl-tRNA synthetase was carried on pHEF200Δ1, whose parent is the *URA3* selectable vector YEp24. All 48 Leu^+ Ura^+ cotransformants of pHET400am and pHEF200Δ1 were found tobe Met^+ Trp^- His^-. Of 40 Leu^+ Ura^+ colonies carryingboth pHET100am and pHEF200Δ1, 37 were Met^+ Trp^+ and weakly His^+, while 3 were scored as Met^+ Trp^+ His^-. Lastly, all 32 Leu^+ Ura^+ cotransformants of pHET120am and pHEF200Δ1 were Met^+ Trp^+ His^+. Thus, the spectrum of suppression is correlated with the amount of tRNA produced by the different constructs (Fig. 6A). Based on the quantitation of tRNA levels given above, the *E. coli* tyrosyl-tRNA synthetase apparently recognizes its tRNA substrate in *S. cerevisiae* cells that carry pHET400am, in which the level of *E. coli* tRNA is estimated as no more than 1% of that of the yeast tyrosine tRNA or as 0.05% or less of that of total cytoplasmic tRNA.

These studies demonstrate that the *E. coli* suppressor is, in fact, able to function with the yeast translational machinery to suppress three yeast amber alleles. Thus, in the absence of the *E. coli* enzyme, the lack of amber suppression may be due to insufficient aminoacylation of the *E. coli* suppressor tRNA.

Cotransformation of a tRNA construction with the vector YEp24 alone does not lead to suppression. Similarly, cotransformants of pHEF200Δ1 plus YEp21 or YCp402 are all Met^- Trp^- His^-. These experiments show that suppression depends on the presence of both the *E. coli* suppressor tRNA and *E. coli* tyrosyl-tRNA synthetase genes (Table 1). An example of the dependence of suppression of the *met8-1* mutation on *E. coli* tyrosine tRNA and tyrosyl-tRNA synthetase is shown in Fig. 6B.

Specificity of suppression, plasmid linkage, and role of copy number. Several control experiments demonstrating the

TABLE 1. Suppression phenotypes of HEY301-129 carrying various *E. coli* suppressor tRNA-encoding vectors and *E. coli* tyrosyl-tRNA synthetase-encoding vectors[a]

Vector 1 (tRNA)	Vector 2 (synthetase)	Phenotype[b]		
		Met	Trp	His
pHET100am	YEp24	−	−	−
YEp21	pHEF200Δ1	−	−	−
pHET100am	pHEF200Δ1	+	+	±
pHET120am	pHEF200Δ1	+	+	+
pHET400am (Cen)[c]	pHEF200Δ1	+	−	−
pHET100am	pHEF300Δ1 (Cen)	+	−	−
pHET400am (Cen)	pHEF300Δ1 (Cen)	−	−	−

[a] Strain HEY301-129 [relevant genotype *leu2-3,112 ura3-1 met8-1*(Am) *trp1-1*(Am) *his4-580*(Am)] was transformed with a plasmid which encodes the *E. coli* tryosine amber suppressor tRNA (vector 1, selectable marker *LEU2*) and a plasmid which encodes the *E. coli* tyrosyl-tRNA synthetase (vector 2, selectable marker *URA3*). Leu$^+$ Ura$^+$ transformants were isolated, patched to YPD, and replica plated to minimal medium supplemented with various amino acids to score for suppression of the three amber alleles.

[b] The Met, Trp, and His phenotypes of the transformants are indicated. Symbols: +, growth in the absence of the amino acid (suppression); −, no growth in the absence of the amino acid (no suppression); ±, intermediate growth (suppression). The Met phenotype was scored after 1 day of growth at 30°C (2 days for strains which carry pHEF300Δ1), and His and Trp phenotypes were scored after 4 days.

[c] Cen indicates a centromere plasmid.

specificity of suppression and plasmid linkage were carried out with strain HEY300-103, which harbors the amber alleles *met8-1* and *trp1-1*. The pattern of suppression observed with pHET120am, pHET100am, and pHEF200Δ1 in tester strain HEY301-129 was confirmed by using the parent HEY300-103. In addition to the amber alleles, strain HEY300-103 carries an *ade1* UGA (opal) suppressible allele and a *lys1-1* (ochre) suppressible allele. Cotransformants of pHET100am plus pHEF200Δ1 or pHET120am plus pHEF200Δ1 were all Met$^+$ Trp$^+$, but were never observed to be Lys$^+$ or Ade$^+$. Thus, suppression by the *E. coli* tRNA appears to be specific for amber codons.

To verify further that suppression depended upon plasmid-encoded *E. coli* suppressor tyrosine tRNA and *E. coli* tyrosyl-tRNA synthetase, we performed segregation analysis on Leu$^+$ Ura$^+$ Met$^+$ Trp$^+$ cotransformants containing either pHET100am plus pHEF200Δ1 or pHET120am plus pHEF200Δ1. Two colonies of each type were grown nonselectively in YPD (rich medium) overnight. Samples from the cultures were plated onto YPD plates and replica plated to synthetic minimal medium supplemented with various amino acids to score for the presence of plasmid and for suppression. For pHET100am-plus-pHEF200Δ1 cotransformants, we observed a total of 149 Leu$^+$ Ura$^-$, 46 Leu$^-$ Ura$^+$, and 23 Leu$^-$ Ura$^-$ colonies from two cultures. These were all Met$^-$ Trp$^-$, whereas 328 Leu$^+$ Ura$^+$ colonies retained the suppressed phenotype (Met$^+$ Trp$^+$). The results for pHET120am-plus-pHEF200Δ1 cotransformants were similar. Thus, whenever either or both plasmids were lost, as indicated by Leu$^-$ and/or Ura$^-$ phenotypes, colonies were Met$^-$ Trp$^-$, demonstrating that suppression requires both plasmids.

Plasmid DNA was isolated from cotransformants which carried either pHET100am plus pHEF200Δ1 or pHET120am plus pHEF200Δ1 and was recovered by transformation of *E. coli*. Of 25 pHEF200Δ1 isolates recovered, 23 (from six different cotransformants) appeared structurally unaltered as determined by restriction analysis. The other two isolates appeared slightly rearranged, but the fragment encoding the synthetase was the expected size. All isolates of pHET100am (two of two from one cotransformant) and pHET120am (three of three from one cotransformant) from *S. cerevisiae* which exhibited suppression also appeared unaltered. The tRNA genes from an isolate of pHET100am and of pHET120am were sequenced, and no changes had occurred in either one. The plasmids isolated from *S. cerevisiae* were used to transform the triple amber tester strain HEY301-129 to demonstrate tRNA- and synthetase-dependent amber suppression, confirming that the plasmids carried the suppression activity. Taken together, the structural analysis and plasmid linkage of the suppression phenotype suggest that no alteration of the suppressor tRNA or rearrangement of the synthetase was necessary for suppression.

Just as reducing the amount of *E. coli* tRNA reduces suppression (see above), lowering the amount of *E. coli* tyrosyl-tRNA synthetase expressed in *S. cerevisiae* lowers the suppression efficiency. We cloned the *Sal*I fragment containing the *E. coli* tyrosyl-tRNA synthetase gene into the *Sal*I site of centromere vector YCp50 (*URA3*) to make pHEF300Δ1. Cotransformation of this plasmid with pHET100am or pHET120am into the amber tester strain gave rise to a Met$^+$ phenotype; however, the transformants took an extra day to achieve full growth. Moreover, there was no suppression of the Trp and His markers as observed when the *E. coli* enzyme was expressed from the higher-copy vector. Expression of both tRNA and synthetase from centromere vectors did not generate enough activity for suppression of any amber marker (Table 1).

DISCUSSION

Several plasmids that direct the production of an *E. coli* tyrosine amber suppressor tRNA in *S. cerevisiae* were constructed. In these constructions, the *E. coli* suppressor tRNA gene was unaltered except for three nucleotide changes in the 3′ noncoding region, which generated a string of seven T residues that may function as a pol III terminator. Transcription of this version of the *E. coli* tyrosine tRNA gene in *S. cerevisiae* contrasts with the finding that an *E. coli* tyrosine tRNA gene was not transcribed when injected into *Xenopus* oocytes (14). Although expression in *S. cerevisiae* of this suppressor alone (at a level just 2.5-fold below that of an average yeast tyrosine tRNA) does not result in suppression of three amber alleles, coexpression with the *E. coli* tyrosyl-tRNA synthetase causes suppression of all three amber alleles. To our knowledge, this is the first demonstration that an *E. coli* tRNA can function with the components of the yeast translational machinery.

The amber suppressible alleles used to detect the heterologous synthetase-tRNA interaction are known to give a positive signal in response to insertion of several different amino acids. If suppression reflects the efficiency of aminoacylation, several interpretations of the behavior of the *E. coli* tRNA in *S. cerevisiae* are possible. For example, the *E. coli* tRNA may lack strong positive elements that are recognized by yeast synthetases, or the *E. coli* tRNA may contain negative elements that prevent nonspecific interactions with yeast charging enzymes, or both. These observations suggest that not all of the determinants the influence the identity of a tyrosine tRNA are conserved between *E. coli* and yeast cytoplasm.

Consistent with this suggestion, crude extracts from *S. cerevisiae* have been shown to aminoacylate *E. coli* tyrosine tRNA to only 5% or not at all (4, 9, 11). Recently a purified preparation of yeast tyrosyl-tRNA synthetase was shown to aminoacylate 15% of pure *E. coli* tyrosine tRNA (C. Flo-

rentz and R. Giegé, personal communication); however, preliminary attempts in this laboratory to aminoacylate the *E. coli* tyrosine tRNA with a partially purified yeast extract have been unsuccessful (V. Trézéguet, unpublished results).

If the determinants for tyrosine identity are partially conserved between yeast cytoplasm and *E. coli*, the lack of heterologous suppression in yeast cytoplasm might be due to context effects, competition with endogenous tRNA (18, 44), limiting amounts of tRNA or endogenous synthetases, or some combination of factors. The features that determine the identity of a tyrosine tRNA have been subjected to limited investigation. Substitutions in the first two positions of the anticodon of the yeast cytoplasmic tyrosine tRNA constructed in vitro are known to decrease the extent of aminoacylation with tyrosine (3). In particular, a pseudouridine (Ψ)-to-U substitution at position 35 reduces the efficiency of in vitro aminoacylation by half. Interestingly, the yeast tyrosine tRNA is the only yeast tRNA known to have pseudouridine in the anticodon (20).

Whether the U in the anticodon of the *E. coli* amber suppressor is modified to Ψ in *S. cerevisiae* is not known. However, the presence of the intron of a yeast tyrosine tRNA ochre suppressor [*SUP6*(o)] is necessary for modification of U-35 to Ψ-35 (20). Similarly, the conversion of C-35 to 5-methylcytosine in a yeast leucine tRNA amber suppressor requires the presence of an intron (43). Both of these undermodified tRNAs are less efficient suppressors in vivo than are their modified counterparts. If an intron is essential for the conversion of U-35 to Ψ-35 in all tRNA contexts, it is unlikely that this modification occurs in the *E. coli* suppressor tRNA, because the suppressor gene lacks an intron. Although the presence or absence of the Ψ-35 or other modifications may influence the suppression efficiency of the *E. coli* tRNA in *S. cerevisiae*, the modifications are not a critical factor, because all three amber alleles are easily suppressed when the *E. coli* synthetase and tRNA are coexpressed.

In contrast to the tyrosine tRNA, the features that determine the identity of *E. coli* alanine tRNA are well defined. A single base pair, G-3–U-70, is the major determinant for alanine identity (17, 30). Hou and Schimmel (19) have recently demonstrated that the G-3–U-70 base pair is also essential for recognition of cytoplasmic *Bombyx mori* (silkworm) and human alanine tRNAs by their respective synthetases in vitro. Furthermore, when expressed in *E. coli*, these tRNAs are active suppressors and are charged only by the *E. coli* alanine tRNA synthetase. This study indicates that the determinants for alanine tRNA identity have been conserved in evolution.

In the case of phenylalanine tRNA, there appears to be a partial conservation of identity determinants between *S. cerevisiae* and *E. coli*. Five nucleotides important for in vitro aminoacylation of yeast phenylalanine tRNA and capable of conferring phenylalanine identity on other tRNAs in vitro have been identified (see reference 36 and references therein). Four of these bases are found in the *E. coli* phenylalanine tRNA; however, the *E. coli* tRNA has a U at position 20 instead of the G found in yeast tRNA. Introduction of G-20 into the *E. coli* tRNA improves aminoacylation by the yeast enzyme 12-fold, resulting in a catalytic efficiency only 2-fold below that of a synthetic yeast phenylalanine tRNA (36). Although the function of the *E. coli* phenylalanine tRNA (U-20) has not been tested in *S. cerevisiae*, it is worth noting that certain *E. coli* alanine tRNA mutants that cannot be aminoacylated in vitro show weak suppression of an *E. coli* amber allele in vivo (38).

A partial conservation of identity between cytoplasmic and *E. coli* tyrosine tRNA might be reflected in vivo in a low efficiency of suppression. Recently, we have observed weak suppression in *S. cerevisiae* by much higher levels of *E. coli* tRNA (20- to 60-fold higher than achieved here) in the absence of *E. coli* synthetase (unpublished results). We are currently developing a system to determine which yeast synthetase(s) aminoacylates this tRNA in vivo.

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