data indicate no difference in the inhibitory effects between different stereoisomers. A weaker inhibitory activity was observed with sphingosines having shorter aliphatic chains. Inconsistency between the results of this paper and theirs may depend on the assay conditions.

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Evidence That a Major Determinant for the Identity of a Transfer RNA Is Conserved in Evolution†

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ABSTRACT: We observed recently that a single G3·U70 base pair in the amino acid acceptor stem of an *Escherichia coli* alanine tRNA is a major determinant for its identity. Inspection of tRNA sequences shows that G3·U70 is unique to alanine in *E. coli* and is present in eucaryotic cytoplasmic alanine tRNAs. We show here that single nucleotide changes of G3·U70 to A3·U70 or to G3·C70 eliminate in vitro aminoacylation of an insect and of a human alanine tRNA by the respective homologous synthetase. Compared to the influence of G3·U70, other sequence variations in $tRNA^{Ala}$ have a relatively small effect on aminoacylation by the insect and human enzymes. In addition, while these eucaryotic tRNAs have nucleotide differences from *E. coli* alanine tRNA, they are heterologously charged only with alanine when expressed in *E. coli*. The results indicate a functional role for G3·U70 that is conserved in evolution. They also suggest that the sequence differences between *E. coli* and the eucaryotic alanine tRNAs at sites other than the conserved G3·U70 do not create major determinants for recognition by any other bacterial enzyme.

The adaptor role of transfer RNAs is manifested through both their codon and amino acid specificities. The codon specificity is determined by the base pairing between the anticodons in tRNAs and the trinucleotide sequences of codons. The amino acid specificity is determined by unique sets of nucleotides that are important for contact with the cognate aminoacyl tRNA synthetases (Schulman & Abelson, 1988; Yarus, 1988; Schimmel, 1989). The conservation of the genetic code in evolution does not demand that the determinants for the identities of tRNAs be conserved. The only requirement is that the same trinucleotide sequences be assigned to each amino acid, and this in principle can be done with determinants for tRNA identity that are unique to each organism.

Early work demonstrated examples of cross-species aminoacylation, misacylation, and lack of aminoacylation (Jacobson, 1971). The results are not easy to interpret in the context of the question of conservation of determinants for tRNA identity. Failure of cross-species aminoacylation, for

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example, could be due to the presence of a blocking or "negative" element that prevents recognition of a conserved major determinant that is present in the heterologous tRNA substrate. Conversely, successful cross-species aminoacylation does not in itself prove that the same determinants are recognized in the heterologous as in the homologous substrate.

For these reasons, we examined whether a major determinant for the identity of an *Escherichia coli* tRNA was impotant for identity in eucaryotes. The alanine specificity of *E. coli* $tRNA^{Ala}$ is dependent upon a G3·U70 base pair in the amino acid acceptor stem (Hou & Schimmel, 1988; Francklyn & Schimmel, 1989). Substitution of G3·C70, A3·U70, or U3·G70 for this base pair completely eliminates aminoacylation with alanine in vitro, even with substrate levels of enzyme (Park et al., 1989). Transfer of G3·U70 to $tRNA^{Cys}$ (Hou & Schimmel, 1988), $tRNA^{Phe}$ (Hou & Schimmel, 1988; McClain & Foss, 1988a), $tRNA^{Lys}$ (McClain et al., 1988), and $tRNA^{Tyr}$ (Hou & Schimmel, 1989) confers upon each the ability to accept alanine. In eucaryotes, G3·U70 is present in all of cytoplasmic alanine tRNAs and, as in *E. coli*, is unique to alanine (Sprinzl et al., 1989). This raises the possibility that the functional role of G3·U70 may have been conserved in evolution. In this paper, we tested the role of this base pair in the recognition of a human (Bunn & Mathews, 1987) and a *Bombyx mori* (Sprague et al., 1977) alanine tRNA to determine whether this element is also essential for alanine specificity of these two eucaryotic alanine tRNAs, which are widely separated from *E. coli* and from each other in evolution.

Materials and Methods

Purified *B. mori* and rat liver alanine tRNA synthetases were provided by Dr. J. D. Dignam (Medical College of Ohio). The *E. coli* alanine enzyme was prepared as described (Hill & Schimmel, 1989).

The genes that encode the human and the *B. mori* $tRNA^{Ala/CUA}$ were synthesized on an Applied Biosystem DNA synthesizer Model 380B in two complementary oligonucleotides. The coding sequence was flanked by an *Eco*RI site at the 5′ end and a *Pst*I site at the 3′ end. Oligonucleotides were purified by the method of Toth and Schimmel (1986) and were phosphorylated by T4 kinase in 100 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$, 7 mM dithiothreitol, and 2 mM ATP, at 37 °C for 1 h. One hundred picomoles of each of the complementary oligonucleotides was mixed, and the concentration of NaCl was adjusted to 0.1 M. The mixture was heated at 80 °C for 3 min and slowly cooled to 25 °C prior to addition to 5 pmol of vector pGFIB, which had been restricted at *Eco*RI and *Pst*I sites, in the ligation reaction. *E. coli* strain *XAC-I* [$F'lac_{373}lacZ_{am}proB^{+}/F^{-}\Delta(lacproB)$ *nalA*, *rif*, $argE_{am}$, *ara*] that harbored a ligated plasmid appeared blue on X-gal indicator plates (Masson & Miller, 1986).

Preparation of plasmid-encoded tRNAs from *E. coli* and the assays for aminoacylation were described by Park et al. (1989). Purification of various DHFR derivatives that resulted from suppression of the fol_{am} gene was described by Normanly et al. (1986a,b).

Results

Aminoacylation of a Human and of a B. mori Alanine tRNA Is Dependent upon G3·U70. The genes coding for the two eucaryotic tRNAs were synthesized de novo and were expressed in *E. coli* from an expression vector (Masson & Miller, 1986). To facilitate characterization of tRNAs made in *E. coli*, we synthesized each gene so as to encode an amber suppressor variant. We therefore replaced the anticodons of these two eucaryotic tRNAs with the amber-reading CUA sequence and introduced a U38 → A in the anticocon loop to enhance suppression efficiency (Raftery & Yarus, 1987) in *E. coli* (Figure 1). As shown below, and as demonstrated previously for the bacterial system (Hou & Schimmel, 1988; Park et al., 1989), these sequence alterations do not prevent aminoacylation by the respective homologous enzyme. To test the role of G3·U70, the G3·C70 and A3·U70 variants of each tRNA were also constructed by oligonucleotide-directed mutagenesis.

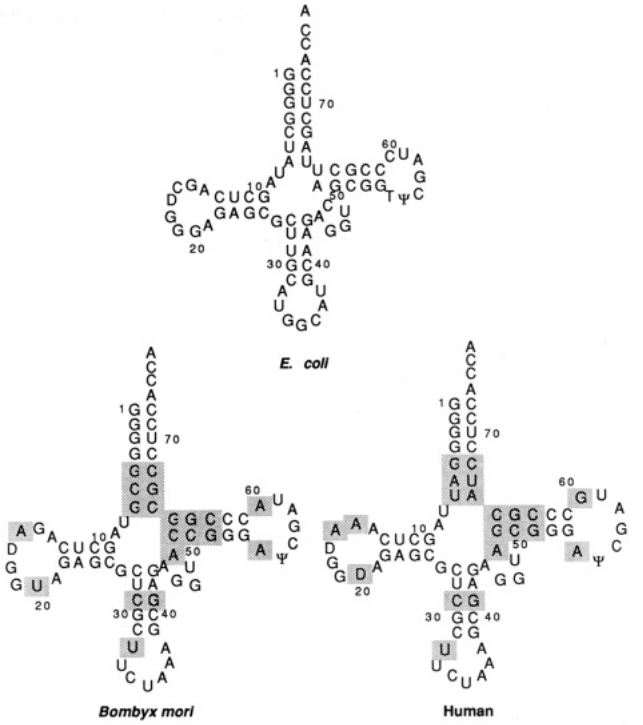

FIGURE 1: Sequence and cloverleaf structure of *E. coli* $tRNA^{Ala/CUA}$, *B. mori* $tRNA^{Ala/CUA}$, and human $tRNA^{Ala/CUA}$. Nucleotides in *B. mori* and in human tRNAs that are different from their *E. coli* counterpart are shaded. Each tRNA was encoded by a construct of plasmid pGFIB in *E. coli* and was purified by elution from a preparative polyacrylamide gel.

Extracts of *E. coli* cells that harbored a suppressor gene were examined for the stable synthesis of the tRNA product by gel electrophoresis (Hou & Schimmel, 1988). In all of the constructs that were examined, we observed an overproduced tRNA species that was absent in a control sample which was prepared from cells without a suppressor gene. These overproduced tRNAs migrated near a purified *E. coli* isoacceptor $tRNA^{Ala/UGC}$ although they varied somewhat in mobility depending on the suppressor sequence and gel conditions. The ability to detect overproduced tRNA products indicates the stable synthesis of transcripts from eucaryotic genes in *E. coli*. We measured by aminoacylation that these suppressors were overproduced 15–20-fold relative to the endogeneous *E. coli* $tRNA^{Ala}$.

Each suppressor was purified by elution from a preparative gel and was tested in vitro for its ability to be aminoacylated with alanine. With highly purified *B. mori* alanine tRNA synthetase, we observed aminoacylation of the wild-type *B. mori* $tRNA^{Ala/CUA}$ but no aminoacylation of the G3·C70 variant (Figure 2a). Because the purified human enzyme is not available, we prepared a crude HeLa cell extract and enriched for aminoacyl tRNA synthetases by chromatography (Pearson et al., 1973). With this extract, we detected alanine incorporation into the human wild-type $tRNA^{Ala/CUA}$ but not into the G3·C70 variant (Figure 2b). Using a purified rat liver alanine enzyme, we confirmed that the wild type, but not the

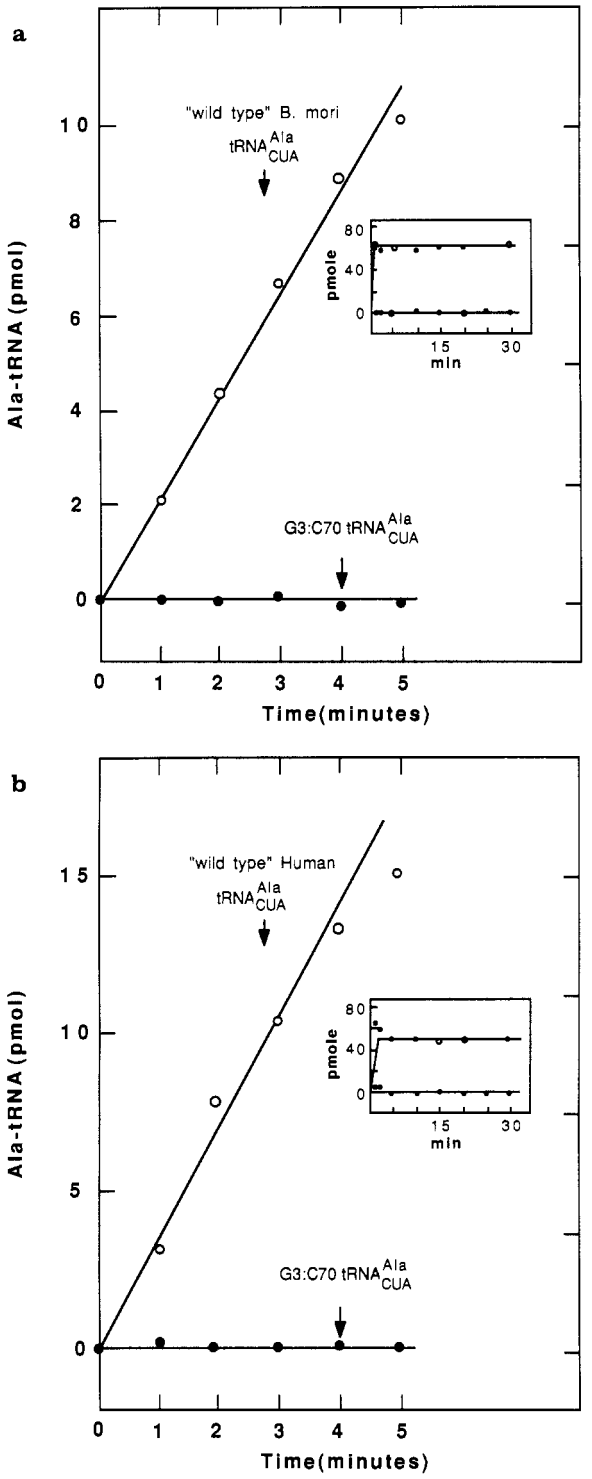

FIGURE 2: Aminoacylation of wild type and the G3·C70 variant of (a) *B. mori* tRNA$^{Ala/CUA}$ and of (b) human tRNA$^{Ala/CUA}$ with alanine by their respective homologous enzymes at 37 °C, pH 7.5. Each tRNA species was incubated at a concentration of 10 μM, and the acid-precipitable counts were measured as described (Schreier & Schimmel, 1972). The purity of these tRNAs collectively varied from 900 to 1400 pmol/OD_{260}. Purified *B. mori* alanine tRNA synthetase was used at an estimated final concentration of 18 nM. HeLa cell extract (7 mg/mL) was used at a final concentration of 14 μg/mL. The inset shows aminoacylation of 1.2 μM "wild type" and G3·C70 variant of *B. mori* tRNA^{Ala} with 100 nM *B mori* alanine tRNA synthetase (a) and of the corresponding human tRNA^{Ala} substrates with 0.7 mg/mL HeLa cell extract (b). The wild-type tRNAs were aminoacylated to 80–100% completion while the G3·C70 variants were inactive.

G3·C70 variant of human alanine tRNA, could be aminoacylated with alanine (not shown). Even during a prolonged incubation with higher levels [5-fold (*B. mori*) and 50-fold (HeLa extract)] of their respective enzymes, the G3·C70 mutants of both eucaryotic tRNAs were not aminoacylated. This behavior has also been observed with *E. coli* alanine tRNA synthetase and the G3·C70 variant of *E. coli* tRNA^{Ala}, whose lack of aminoacylation has been shown to be due to a kinetic defect that is prior to product formation and release (Park et al., 1989). Similarly, the A3·U70 variants of *B. mori* tRNA$^{Ala/CUA}$ and of human tRNA$^{Ala/CUA}$ gave no aminoacylation when incubated with either catalytic or higher levels of their respective enzymes. We conclude that aminoacylation of *B. mori* tRNA$^{Ala/CUA}$ and of human tRNA$^{Ala/CUA}$ is dependent upon G3·U70. Because replacement of this base pair with either A3·U70 or G3·C70 completely eliminates aminoacylation, the G3·U70 base pair must have a major role in the recognition of *B. mori* and of human alanine tRNAs.

G3·U70 Is a Major Determinant in Cross-Species Aminoacylation. The purified *B. mori* alanine tRNA synthetase also aminoacylated the wild-type human tRNA$^{Ala/CUA}$ with alanine (see below). Conversely, HeLa cell extract and the purified rat liver alanine enzyme charged the wild-type *B. mori* tRNA$^{Ala/CUA}$ with alanine (see below). Both wild-type tRNAs were also substrates for the *E. coli* alanine tRNA synthetase. However, none of these enzymes recognized the G3·C70 variants of any heterologous alanine tRNA (not shown). Thus, even in the context of different tRNA sequences, the G3·U70 base pair is a major determinant for the bacterial, insect, and mammalian enzymes.

For a given enzyme, there are context effects among the different alanine tRNA substrates. While the three wild-type suppressors were aminoacylated by the purified *B. mori* enzyme, we observed differences in the initial rates. Under the standard assay conditions, the *B. mori* enzyme reacted rapidly and efficiently with the two eucaryotic tRNAs but more slowly with the *E. coli* tRNA. We detected the highest relative rate with the *B. mori* tRNA^{Ala} (100%), followed by the human tRNA^{Ala} (89%) and the *E. coli* tRNA^{Ala} (51%) (not shown). With the same conditions, the rat liver enzyme had a greater efficiency with *B. mori* tRNA^{Ala} (100%) and human tRNA^{Ala} (88%) than with the *E. coli* counterpart (50%) (not shown). In contrast, the *E. coli* enzyme reacted well with *E. coli* tRNA^{Ala} (100%) and showed progressively less activity with *B. mori* tRNA^{Ala} (69%) and human tRNA^{Ala} (48%) (Figure 3). These results show that, for a given enzyme, the discrimination between procaryotic and eucaryotic substrates is relatively small when compared to the discrimination of G3·U70 from G3·C70 and A3·U70 variants by all of these enzymes. Thus, while there are context effects, the nucleotides in human and in *B. mori* alanine tRNAs that are different from those in *E. coli* tRNA^{Ala} (shown by shading in Figure 1) are not essential for aminoacylation by the homologous enzymes.

Despite Context Effects, Human and B. mori Alanine tRNAs Are Only Recognized by E. coli Alanine Enzyme in Vivo. The nucleotides that differ between the eucaryotic and *E. coli* alanine tRNAs could be determinants for recognition by other synthetases in *E. coli*. The competitive advantage of the *E. coli* alanine enzyme with the eucaryotic tRNAs would be diminished by the context effects that are evident in Figure 3. To examine the suppression behavior and aminoacylation specificity of the eucaryotic alanine tRNAs in *E. coli*, we first introduced the genes for these tRNAs into *E. coli* strain *XAC-I*. This strain carries an amber mutation in *lacZ* (Masson & Miller, 1986). Suppression of this amber mutation leads to synthesis of β-galactosidase, the activity of which can be measured and compared to that of a wild-type

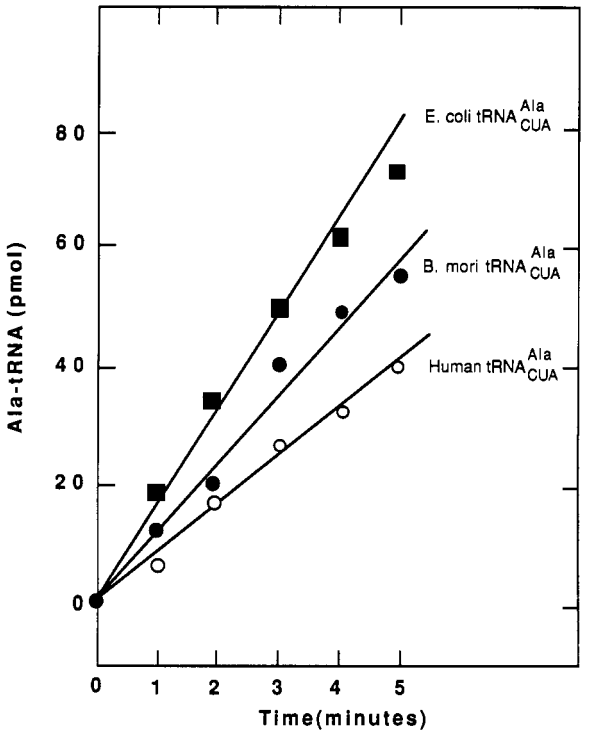

FIGURE 3: Aminoacylation of *E. coli* $tRNA^{Ala/CUA}$, *B. mori* $tRNA^{Ala/CUA}$, and human $tRNA^{Ala/CUA}$ at 37 °C, pH 7.5, with 5 nM *E. coli* alanine tRNA synthetase.

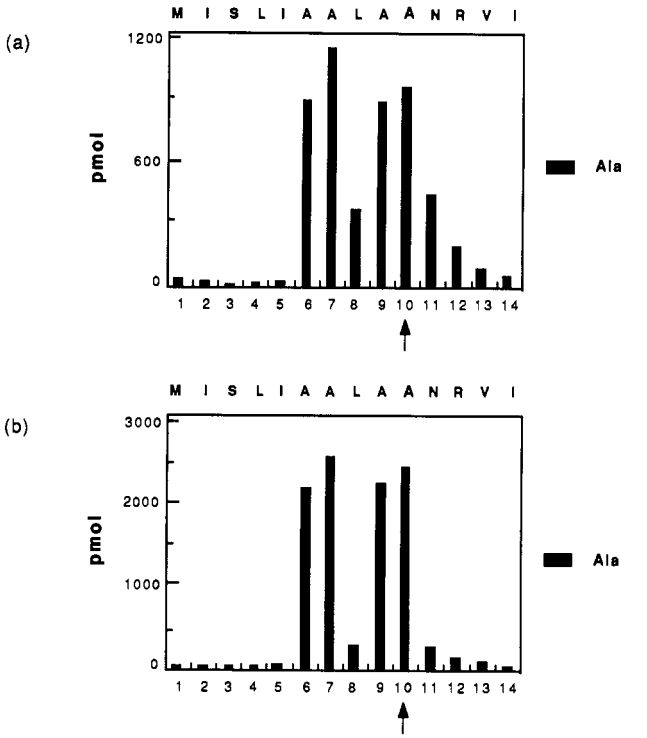

FIGURE 4: Amino acid sequence analysis of the first 14 residues of DHFR. The fol_{am} allele was created by altering the *fol* gene to code for amber–asparagine at codons 10 and 11, and the variants of DHFR that resulted from suppression were purified (Normanly et al., 1986a,b). The picomolar yields of alanine are plotted versus the residue number for the fol_{am} gene products whose amber codons were suppressed by (a) *B. mori* $tRNA^{Ala/CUA}$ and (b) human $tRNA^{Ala/CUA}$. The sequence of the gene product is given across the top of each diagram.

strain. We determined that both eucaryotic alanine tRNAs were active suppressors in *E. coli*. The suppression efficiencies of *B. mori* and of human $tRNA^{Ala/CUA}$ in *XAC-I* were respectively 3.1% and 2.8%. These values are at least 5 times lower than that of the *E. coli* $tRNA^{Ala/CUA}$ which has an efficiency of 20% (Hou & Schimmel, 1989).

To determine whether these two eucaryotic alanine tRNAs would elicit interactions with other synthetases in *E. coli*, we introduced the suppressor genes into an *E. coli* strain which harbored an amber mutation at position 10 of a plasmid-encoded *fol* gene [dihydrofolate reductase (DHFR)] (Normanly et al., 1986a,b). For each suppressor, we purified the protein product of *fol* and subjected it to sequence analysis. The results are shown in Figure 4. Analysis of the first 14 residues of DHFR shows that both suppressors inserted alanine at position 10 (Figure 4a,b). At this position, no other amino acid was present as more than 4% of alanine. Besides alanine, a few amino acids (such as aspartic acid, glycine, and lysine) showed a slight increase in molar yield from position 9 to position 10 (not shown). These amino acids, however, were detected as below 3% of the alanine signal. Therefore, within the limits and sensitivity of protein sequencing, alanine was the only amino acid inserted at position 10.

Because cysteine is normally destroyed during Edman degradation, sequence analysis did not rule out the presence of cysteine at position 10. To check whether bacterial determinants for cysteine were present in these two eucaryotic alanine tRNAs, we examined their ability to suppress an amber codon at position 68 of the gene responsible for ampicillin resistance (Amp^R) (Normanly et al., 1986a). At position 68, the wild-type amino acid is serine (Knott-Hunziker et al., 1979), and the only other amino acid that is known to be functional is cysteine (Sigal et al., 1982). The results of suppression were negative for both suppressors, indicating that they were not aminoacylated with cysteine.

Our results suggest that alanine tRNAs of *B. mori* and of human retain their amino acid specificity for alanine in *E. coli*. Despite their nucleotide differences from *E. coli* $tRNA^{Ala}$ and despite context effects, we detect recognition only by alanine tRNA synthetase. The implication is that major determinants for the identities of other bacterial tRNAs were not acquired in the evolution of the *B. mori* and human alanine tRNAs.

Discussion

The molecular basis by which G3·U70 is distinguished from other nucleotide combinations at the 3·70 position is not known. Park et al. (1989) showed that, for the *E. coli* enzyme, both k_{cat} and K_m play a role in the discrimination of bases of 3·70. It has been proposed that a helix variation caused by the G·U pair and not the G3·U70 base pair per se is the essential feature recognized by the enzyme (McClain et al., 1988). The eucaryotic tRNAs investigated here have not evolved alternatives such as A3·C70 or U3·G70 which might also create a helix variation. Moreover, among other base replacements, substitution of U3·G70 for G3·U70 also inactivates *E. coli* $tRNA^{Ala}$ for aminoacylation in vitro, even with substrate levels of enzyme (Park et al., 1989). Thus, while there may be some effect on $tRNA^{Ala}$ recognition imparted by a helix variation in vivo (McClain et al., 1988), the G3·U70 base pair is the only one known to confer efficient aminoacylation in vitro (Schimmel, 1989). Further investigation of additional mutant tRNA substrates and eventual structural analysis of the enzyme–tRNA complex [cf. Frederick et al. (1988)] will provide further clarification.

Although we show here that the major determinant for the identity of alanine tRNA is conserved in evolution, it remains to be seen whether the identity elements of other tRNAs are similarly conserved. For example, the phenylalanine specificity of yeast $tRNA^{Phe}$ is determined by five nucleotides (Sampson et al., 1989), four of which (three nucleotides in the anticodon

sequence and the fourth nucleotide from the 3′ end) are found in all sequenced phenylalanine tRNAs and their genes (Sprinzl et al., 1989). It is not yet known whether the recognition of a phenylalanine tRNA other than yeast is influenced by these four nucleotides. However, there are data which suggest conservation of a functional role for position 20 in *E. coli* (McClain & Foss, 1988b) and in yeast (Sampson et al., 1989), although the nucleotide itself is different (G20 in yeast and U20 in *E. coli*).

Our studies do not address whether specific base modifications affect the homologous-enzyme recognition of the eucaryotic tRNAs. *B. mori* alanine tRNA contains modifications of nucleotides (mostly methyl derivatives) in the D-stem, anticodon stem–loop, and TΨC stem–loop that are absent in *E. coli* alanine tRNA (Sprague et al., 1977). Because the *B. mori* enzyme can aminoacylate *E. coli* alanine tRNA, these differences in base modifications do not appear to be significant relative to the requirement for a G3·U70 base pair.

The conservation of a functional role for G3·U70 may indicate that the domain in alanine tRNA synthetase that makes contact with G3·U70 shares some sequence/structural similarity among different species. It is noteworthy that antibodies raised against *B. mori* alanine tRNA synthetase react with an epitope of the *E. coli* enzyme that is believed important for aminoacylation (Regan et al., 1986). Further molecular dissection of the *E. coli* and *B. mori* enzymes, and of enzymes from other organisms, is needed to elucidate the protein determinants for recognition of tRNA and to determine whether they are conserved in evolution.

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