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Parameters for the Molecular Recognition of Transfer RNAs†

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Transfer RNAs are highly differentiated nucleic acids comprised of 74–93 nucleotides that are folded into a compact three-dimensional pattern which is believed to accommodate most of the known tRNA sequences (Sprinzl, 1987). The molecules are differentiated from each other according to their amino acid acceptance. This is determined in the two-step aminoacylation reaction whereby an amino acid is activated by its cognate aminoacyl-tRNA synthetase, which catalyzes formation of a tightly bound aminoacyl adenylate; this complex than reacts with the 2′- or 3′-hydroxyl of a tRNA to form the aminoacylated species. After this, aminoacyl-tRNAs react with components of the translation apparatus that recognize features common to all tRNAs and enable amino acids to be inserted into growing polypeptide chains through the precise base-pairing interaction of trinucleotide anticodons (within tRNAs) with codons in messenger RNAs. Thus, the translation of mRNAs into polypeptides of defined sequences is a manifestation of the genetic code, but the code itself is based on the molecular recognition of transfer RNAs by aminoacyl-tRNA synthetases. This system of protein–nucleic acid recognition connects each amino acid with a trinucleotide sequence (anticodon) within the tRNA.

Because of degeneracy in the genetic code, whereby up to six trinucleotide sequences may code for a specific amino acid, there can be several tRNAs that are specific for a given amino acid. An example is serine, which in *Escherichia coli* has at least five distinct tRNA species that collectively recognize the six different codons for that amino acid (Sprinzl et al., 1987). These tRNAs differ not only in their anticodons but also in other parts of their respective sequences. These tRNA isoacceptors are generally recognized by the one aminoacyl-tRNA synthetase which is specific for that amino acid, however (Schimmel & Soll, 1979). This implies that, for at least some of the enzymes, the anticodon is not the primary determinant for recognition.

Unlike tRNAs that have a common structural motif and typically vary in length by not more than 20%, the aminoacyl-tRNA synthetases are diverse proteins with four different types of quaternary structures (α, α_2, $\alpha_2\beta_2$, and α_4) and subunit sizes that range from 334 to over 1000 amino acids (Schimmel, 1987). Although leucine, valine, and isoleucine tRNA synthetases have some sequence similarities (Heck & Hatfield, 1988), several of the enzymes have sequences which are not similar to that of any other synthetase. The enzymes arose early in evolution, probably appearing with the earliest life forms, and may never have been constrained to interact with elements of the protein synthesis apparatus other than tRNAs. In some cases, they have acquired additional biological functions. These considerations may explain in part the apparent uniqueness of the sequences and quaternary structures of many of the enzymes. However, similar structural motifs can in principle be assembled from diverse sequences, and there is evidence for a structural relatedness of portions of methionine and tyrosine tRNA synthetases, which have little sequence similarity (Blow et al., 1983).

The basis for the recognition of tRNAs by aminoacyl-tRNA synthetases has been difficult to solve [recent commentaries and summaries include de Duve (1988), Schulman and Abelson (1988), RajBhandary (1988), and Yarus (1988)]. There are significant differences from the system of recognition of DNA sequence elements by gene regulatory proteins. For protein–DNA complexes, the dissociation constant can be as small as 10^{-12} M, so that the complexes have long lifetimes (Pabo & Sauer, 1984). For synthetase–tRNA complexes, the dissociation constants at pH 7.5 are on the order of 10^{-6} M, which is comparable to the dissociation constant or Michaelis constant for an enzyme–small molecule complex (Schimmel & Soll, 1979). This means that the enzyme–tRNA complexes have short lifetimes, which facilites rapid turnover. The relatively high enzyme–tRNA dissociation constant limits the degree to which discrimination can be achieved at the binding step. There is a second step in which specificity can be manifested, however. Because tRNA is a substrate for the enzyme, there can be discrimination during the transition state of aminoacylation, which is expressed through the catalytic parameter k_{cat}. Early work suggested that this played a role

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in the overall recognition process (Schimmel & Soll, 1979).

The tRNA molecule is folded, like many proteins, into a globular structure where some elements which are dispersed in the sequence are brought into close proximity (Kim et al., 1974; Robertus et al., 1974). Dissection of tRNAs into smaller pieces, as has been done in successfully identifying recognition sites in linear DNA molecules, sacrifices many of the structural features that only are present in the intact tRNA. The relatively short lifetimes of the synthetase–tRNA complexes make it difficult to obtain, from a nuclease digestion, a nuclease–resistant protein–nucleic acid complex—an approach that has also been successfully applied with the more stable DNA–protein complexes. Early work defined sites on tRNAs that are in close contact with bound synthetases [e.g., by using cross-linking among other approaches (Schimmel, 1977)] and investigated the basis for the mischarging of certain tRNAs when reacted with synthetases from another, heterologous organism [summarized in Schimmel and Soll (1979)]. The early application of an in vivo amber suppression assay also afforded an opportunity to explore determinants important for the recognition of tRNAs, although definitive conclusions were not reached at that time and the complexity of the recognition problem was highlighted (see below).

The recent availability of procedures for mutagenesis and expression of tRNAs in vivo, and for synthesis of tRNAs and variants in vitro, has stimulated a new generation of experiments. While much of the recent work has utilized in vivo amber suppression assays to study molecular recognition, the interpretation of some of these experiments is limited when there are no in vitro data. At the same time, in vitro experiments have their own limitations which can only be overcome by investigation of variant tRNAs in the context of all of the synthetases and tRNAs in vivo. Summarized below are recent experiments on the molecular recognition of transfer RNAs, with some consideration of binding and catalytic parameters that enable tRNAs to be distinguished from each other.

Locating Determinants for Identity

Amber Suppression Assay. Early experiments utilized the *E. coli supF* amber suppressor, which is based on the sequence of a tyrosine tRNA with the anticodon altered from GUA to CUA so that the UAG amber codon is recognized [summarized by Ozeki et al. (1980)]. This suppressor inserts tyrosine at amber codons, which demonstrates that the first position of the anticodon is not an essential determinant for the identity of a tyrosine tRNA. Mutants of *supF* that insert glutamine were isolated. The interpretation was not straightforward because some of the mutations that resulted in aminoacylation with glutamine did not create nucleotides that are in the analogous location of any *E. coli* $tRNA^{Gln}$ species (Figure 1a). Subsequently, it was demonstrated that simple alteration of the anticodon of $tRNA^{Trp}$ from CCA to CUA created an amber suppressor that inserts glutamine (Figure 1b). These results demonstrated that amino acid acceptance could be manipulated by simple mutations. One interpretation was that the mutations disturbed the interaction with the cognate enzyme and that glutamine tRNA synthetase had a relaxed specificity which was manifested on tRNA substrates that were less competitively aminoacylated by their cognate enzyme.

Conversion of a Leucine into a Serine Transfer RNA. Although difficulties of interpretation raised concerns about the early work on amber suppressors with altered amino acid acceptance capability, the approach has clear advantages. By restriction of amino acid substitutions of mutant suppressor tRNAs to amber codons, the possibility is removed for deleterious substitutions in vivo in the translated regions of

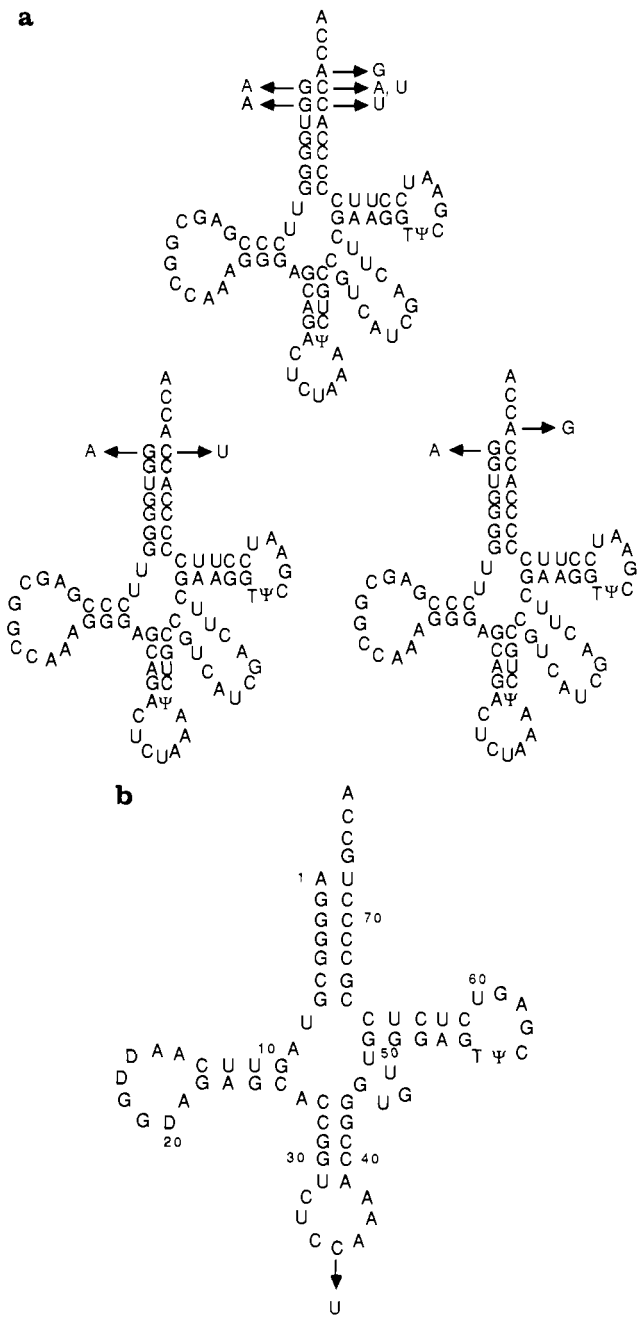

FIGURE 1: (a) Mutations in *E. coli* $tRNA^{Tyr/CUA}$ that cause insertion of glutamine at amber codons. The nucleotide changes are concentrated in the acceptor helix and in some cases do not recreate sequences found in any *E. coli* $tRNA^{Gln}$. The upper diagram shows the locations of single nucleotide substitutions that enable $tRNA^{Tyr/CUA}$ to be aminoacylated with glutamine, while the lower two diagrams show, separately, individual mutants that have two base changes. Data are summarized from Smith et al. (1970), Hooper et al. (1972), Shimura et al. (1972), Smith and Celis (1973), Celis et al. (1973), Ghysen and Celis (1974), Inokuchi et al. (1974), and Ozeki et al. (1980). (b) A single nucleotide substitution in *E. coli* $tRNA^{Trp}$ enables it to insert glutamine at amber codons. The substitution creates an amber CUA anticodon (Yaniv et al., 1974). See also Soll (1974), Celis et al. (1976), and Yarus et al. (1977).

mRNAs for cellular proteins. The approach is limited to those tRNAs whose amino acid acceptance is not altered by change of the anticodon to CUA. One example is serine tRNA, where there are six known isoacceptors (five encoded by the *E. coli* genome and one by phage T_4) that can be aminoacylated by the *E. coli* serine tRNA synthetase and that have anticodons which include UGA, CGA, GGA, and GCU. These anticodons collectively vary all three positions, and furthermore, a

change to CUA does not change the identity of this tRNA.

Normanly et al. (1986a) examined the 6 serine tRNAs and concluded that, apart from the bases that are conserved in all tRNAs, only 12 were held in common. These 12 nucleotides, which are located in the acceptor stem and dihydrouridine stem and loop, were transferred into a leucine tRNA isoacceptor (with a CUA amber anticodon). The resulting tRNA suppresses a serine-requiring amber allele at codon 68 of β-lactamase. The suppression efficiency of the strong parent leucine amber suppressor is 60%, and the transformed species with 12 substitutions has an efficiency of 0.5–1%. Direct information on the nature of the amino acid(s) inserted by the transformed $tRNA^{Leu/CUA}$ was obtained by protein sequence analysis of a suppressed dihydrofolate reductase gene which has an amber mutation at codon 10. This showed that serine was inserted, with a possibility of, but little evidence for, minor amounts of valine and/or leucine. No glutamine was detected at the position corresponding to the amber codon. Thus, the transformation of a strong leucine-inserting suppressor into a weak serine-inserting suppressor was achieved. Further work has focused on defining the minimal subset of nucleotides from among the 12 defined in these experiments, which are sufficient to confer serine acceptance, and determining whether that subset will function in the context of other tRNA sequences (Schulman & Abelson, 1988).

A Single Base Pair Is a Major Determinant of the Identity of an Alanine tRNA. Alanine tRNA synthetase has been extensively studied by biochemical and genetic approaches, and the segment important for tRNA binding has been delineated (Schimmel, 1987, 1989). In order to define further the amino acids that are critical for tRNA recognition, a large population of $tRNA^{Ala}$ variants were created by site-directed mutagenesis and tested for recognition by alanine tRNA synthetase (Hou & Schimmel, 1988). Those that are defective in recognition were to be used to isolate second-site revertants in the enzyme which compensate for the defect in the tRNA. In the course of this work it was discovered that a single base pair is a major determinant of the identity of $tRNA^{Ala}$.

A population of 28 mutant tRNAs were created that collectively varied over half of the nonconserved nucleotides. A $tRNA^{Ala/CUA}$ amber suppressor was used for these experiments. The sequence of this tRNA is based on that of $tRNA^{Ala/GGC}$ [Mims et al., 1985; cf. Normanly et al. (1986b) and Masson and Miller (1986)]. This suppressor inserts alanine, even though all three anticodon nucleotides have been changed. The mutant tRNAs, which in some cases had as many as five substitutions within a single species, were first introduced on a multicopy plasmid and checked for suppression of a *trpA(UAG234)* amber allele. There is evidence that this allele is suppressed by insertion of glycine or alanine but not by other amino acids (Murgola & Hijazi, 1983). Any mutant that did not suppress the *trpA(UAG234)* allele was then checked to determine whether a stable tRNA was made. Those examples of a sup^- phenotype for which the overproduced tRNA was not evident were not considered further. Of particular interest are those sup^- species that are not defective in tRNA biosynthesis.

This screen and analysis yielded only one site in the entire molecule where mutations resulted in a tRNA which has a sup^- phenotype on the *trpA(UAG234)* amber allele and which is clearly synthesized as a stable tRNA. This involved replacements of the G3·U70 base pair in the amino acid acceptor helix with A3·U70 or G3·C70 (Figure 2). In vitro aminoacylation measurements confirmed that the mutant tRNAs were not aminoacylated with catalytic amounts of purified alanine tRNA synthetase (Hou & Schimmel, 1988).

The G3·U70 base pair was introduced into $tRNA^{Cys/CUA}$ and $tRNA^{Phe/CUA}$. Each of these tRNAs has a C3·G70 base pair and differs by 38 ($tRNA^{Cys/CUA}$) and 31 ($tRNA^{Phe/CUA}$) nucleotides from $tRNA^{Ala/CUA}$. Introduction of the G3·U70 base pair into each of these tRNAs confers the ability to accept alanine in vivo. In the case of G3·U70 $tRNA^{Cys/CUA}$, only alanine was detected at the position of the suppressed amber codon in dihydrofolate reductase (Hou & Schimmel, 1988). This raises the possibility that the substitution at position 3·70 has perturbed a determinant for the cysteine tRNA synthetase in addition to conferring a determinant for alanine tRNA synthetase. Aminoacylation of G3·U70 $tRNA^{Cys/CUA}$ with purified alanine tRNA synthetase was demonstrated. The G3·U70 $tRNA^{Phe/CUA}$ species is aminoacylated with both alanine and phenylalanine in vivo. This suggests that the determinants for the identity of phenylalanine tRNA are located, at least in part, elsewhere.

The results indicate that the G3·U70 base pair is a major determinant for the identity of an alanine tRNA. The result of introducing a G3·U70 base pair into $tRNA^{Phe}$ was confirmed by McClain and Foss (1988a). These authors also did experiments with a variant of a glycine tRNA (which largely is aminoacylated with glutamine in vivo). Introduction of the G3·U70 base pair into this tRNA does not confer acceptance of alanine. This may mean that, at least in the context of the variant sequence, the glutamine enzyme competes more effectively than the alanine enzyme and, additionally or alternatively, there are negative determinants in the variant tRNA which block interaction with the alanine enzyme (see Concluding Remarks).

A mutant lysine *missense* suppressor that inserts glycine and/or alanine at lysine codons has been reported (Prather et al., 1984). This suppressor also is believed to insert lysine. The mutation creates a G3·U70 base pair, and while the insertion of alanine was not established, Prather et al. (1984) suggested that this was likely. The aforementioned results and recent experiments with a lysine tRNA amber suppressor (McClain et al., 1988) support this possibility.

Among the published *E. coli* tRNA sequences the G3·U70 base pair is unique to alanine. Further mutational analysis may uncover additional nucleotides that affect recognition by the alanine enzyme. If such nucleotides are uncovered, then it will be important to determine whether transfer of any of them into another tRNA framework will confer identity for alanine.

Role of the Anticodon in the Recognition of Methionine and Valine tRNAs. In vitro aminoacylation experiments have indicated that, for several tRNAs, single base changes in the anticodon affect the rate of aminoacylation. The availability of T7 RNA polymerase and synthetic DNA templates for transcription has made possible the preparation of synthetic tRNAs of any sequence whatsoever (Sampson & Uhlenbeck, 1988). This has afforded an opportunity to make substrates with varied anticodon sequences and to evaluate quantitatively the effect on aminoacylation.

The CAU anticodon of *E. coli* methionine tRNA is known to be important for in vitro aminoacylation with *E. coli* methionine tRNA synthetase (Schulman & Pelka, 1983, 1988). (This enzyme also aminoacylates initiator and elongator methionine tRNAs from other prokaryotes and from eukaryote organelles, all of which have the CAU anticodon.) At concentrations of tRNA that are below the Michaelis constant K_m, the initial rate of aminoacylation is given by $(k_{cat}/K_m)(E)_0(tRNA)_0$, where $(E)_0$ and $(tRNA)_0$ are total enzyme

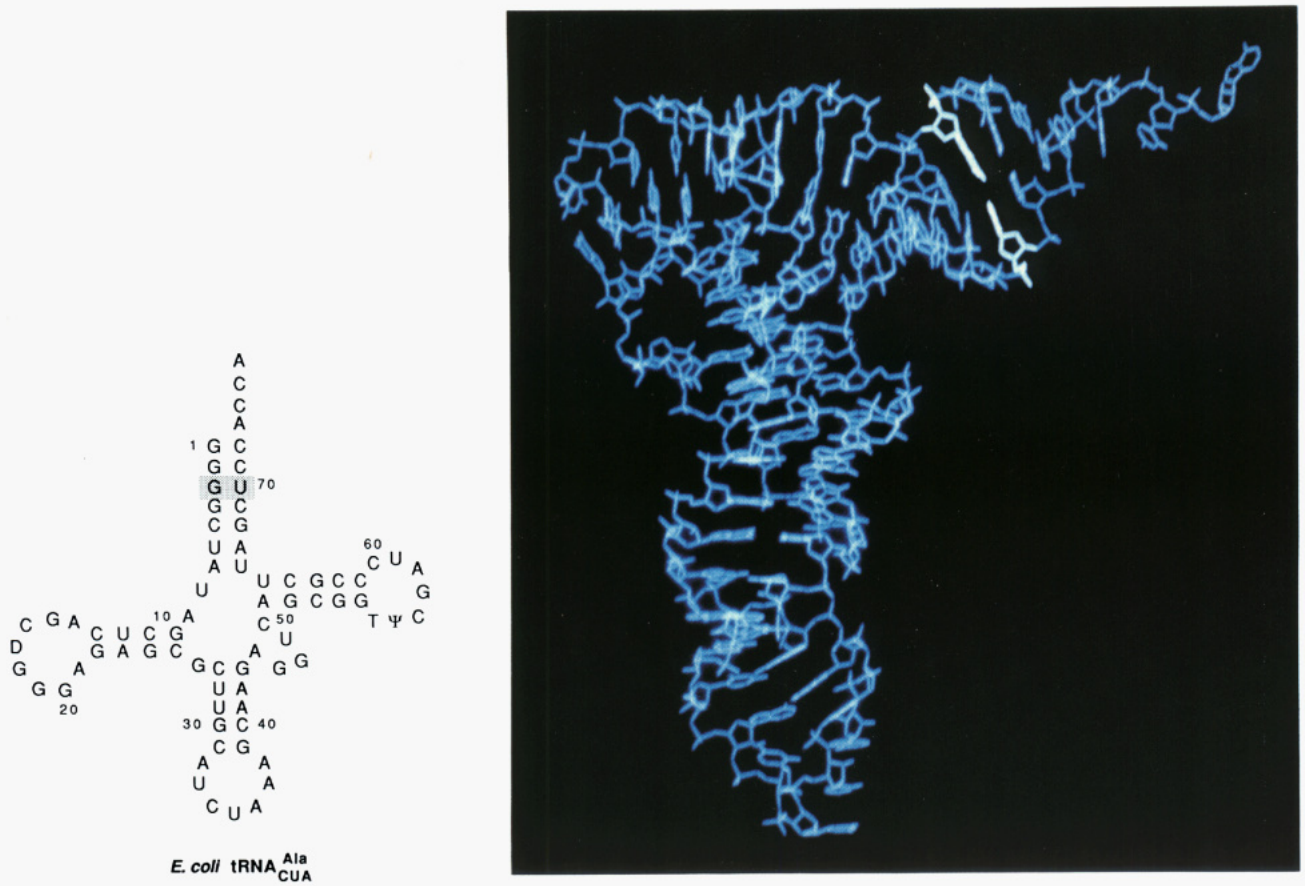

FIGURE 2: (a, left) Nucleotide sequence and cloverleaf structure of $tRNA^{Ala/CUA}$. The sequence is based on $tRNA^{Ala/GGC}$ (Mims et al., 1985) but where the GGC anticodon has been changed to CUA and a U38 → A substitution has been introduced to improve the efficiency of amber suppression (Raftery & Yarus, 1987). The G3·U70 base pair is highlighted. This base pair is a major determinant of the identity of an alanine tRNA (Hou & Schimmel, 1988). (b, right) Depiction of the three-dimensional structure of $tRNA^{Ala/CUA}$ with the G3·U70 base pair highlighted in white. The sequence of $tRNA^{Ala/CUA}$ was built into the known coordinates of yeast $tRNA^{Phe}$ (obtained from Brookhaven Data Bank) utilizing the PS300 FRODO program (Bush et al., 1987).

and tRNA concentrations, respectively, and k_{cat} is the turnover number (unimolecular rate constant). The parameter k_{cat}/K_m has units of a second-order rate constant. Table Ia shows that the relative k_{cat}/K_m is reduced 4–5 orders of magnitude by substitutions at the first position of the anticodon (Schulman & Pelka, 1988). The large effect of substitutions at the first position demonstrates its importance, but it alone is not sufficient for recognition by the methionine enzyme, however. None of the amber suppressors (CUA anticodon) and natural tRNAs with a first position C are known to insert methionine in vivo.

An even larger effect is found when the anticodon is reversed from CAU to UAC. The UAC anticodon corresponds to that for a valine tRNA. Replacement of that anticodon in $tRNA_1^{Val}$ with CAU confers methionine acceptance on $tRNA_1^{Val/CAU}$ (Table Ia). The efficiency of aminoacylation is quantitatively close to that of $tRNA^{Met}$. In a reciprocal experiment, the CAU anticodon of elongator $tRNA_m^{Met}$ was replaced by UAC, and aminoacylation with valine tRNA synthetase was attempted. In this case the anticodon replacement converts the hybrid $tRNA^{Met/UAC}$ into a substrate for valine tRNA synthetase, with a relative k_{cat}/K_m that is 10-fold less than that of $tRNA^{Val}$ (Table Ib). Thus the CUA → UAC substitution simultaneously eliminates aminoacylation with the methionine enzyme while conferring acceptance of valine. The $tRNA^{Met/UAC}$ and $tRNA^{Val/CAU}$ species have been checked for aminoacylation in vitro with glutamic, glutamine, lysine, isoleucine, and phenylalanine tRNA synthetases. These enzymes aminoacylate neither $tRNA^{Met/UAC}$ nor $tRNA^{Val/CAU}$ (Schulman & Pelka, 1988).

Table I: Relative Values of k_{cat}/K_m for Aminoacylation with Methionine and Valine of Anticodon Sequence Variants of $tRNA_m^{Met}$ and $tRNA^{Val}$

tRNA	rel k_{cat}/K_m
(a) Aminoacylation with Methionine[a]	
natural $tRNA^{Met}$	1.0
synthetic $tRNA^{Met}$ (CAU)	0.5
synthetic $tRNA^{Met}$ (UAU)	0.0001
synthetic $tRNA^{Met}$ (GAU)	0.00001
synthetic $tRNA^{Met}$ (UAC)	0.0000001
synthetic $tRNA^{Val}$ (CAU)	0.8
(b) Aminoacylation with Valine[b]	
natural $tRNA^{Val}$	1.0
synthetic $tRNA^{Val}$ (UAC)	0.4
synthetic $tRNA^{Met}$ (CAU)	0.000002
synthetic $tRNA^{Met}$ (UAU)	0.00001
synthetic $tRNA^{Met}$ (UAC)	0.04

[a] Data were obtained with purified *E. coli* methionine tRNA synthetase at pH 7.5, 37 °C, and are taken from Shulman and Pelka (1988). [b] Data were obtained with purified *E. coli* valine tRNA synthetase at pH 7.5, 37 °C, and are taken from Schulman and Pelka (1988).

The data presented in Table I suggest that the anticodon has an important role in defining the identity of valine and methionine tRNAs and that the quantitative effects of simple nucleotide substitutions are large. In order to evaluate the role of the anticodon more fully, and to evaluate the significance

of the anticodon nucleotide replacements in the presence of all of the enzymes and tRNAs, experiments are being attempted in vivo. This necessitates special techniques because the amber suppression system (which requires a CAU anticodon) obviously cannot be used in these circumstances.

Recognition of E. coli Glutamine tRNA. The anticodon (CUG and UUG) has been implicated as a recognition site for glutamine tRNA synthetase. This apparently explains why certain amber suppressor tRNAs (CUA anticodon) mischarge at least partially with glutamine. The central U of the anticodon is believed to have an important role. Rogers and Soll (1988) have manipulated a serine-inserting $tRNA^{Ser/CUA}$ amber suppressor to examine one way to achieve discrimination of glutamine from serine tRNA synthetase. Because of the CUA anticodon, this suppressor potentially could be converted to a glutamine tRNA. A similar example had been previously demonstrated by isolation of mutations in the acceptor helix of a *supF* $tRNA^{Tyr/CUA}$ (Figure 1a).

The three base pairs at the end of the acceptor helix are implicated in recognition by the serine tRNA synthetase (Normanly et al., 1986a). The sequence at the beginning of the acceptor helix of $tRNA_1^{Ser}$ is G1·C72:G2·C71:A3·U70. This was changed to **U1·A72**:G2·C71:**G3·C70**, where the altered bases are indicated in bold type. These substitutions recreate the sequence of the first three base pairs of $tRNA_2^{Gln}$. While $tRNA^{Ser/CUA}$ inserts serine, the U1·A72:G3·C70 $tRNA^{Ser/CUA}$ inserts over 90% glutamine and about 5% serine in vivo (Rogers & Soll, 1988). One interpretation of this result is that substitution of four nucleotides has disrupted the interaction with the serine tRNA synthetase, thus making possible a more efficient competition by the glutamine enzyme. Additionally or alternatively, the substitutions have improved the interaction with glutamine tRNA synthetase. Analytical aminoacylation measurements would clarify this question, and further substitution and analysis will have to be done in order to define the determinants for the identity of a glutamine tRNA.

Determinants for Recognition of Yeast Phenylalanine tRNA. An in vitro analysis has been used to evaluate nucleotides important for recognition of *Saccharomyces cerevisiae* $tRNA^{Phe}$ by the homologous phenylalanine tRNA synthetase. Initial experiments established that replacements of any of the three anticodon nucleotides decreased k_{cat}/K_m by a factor of 3–10-fold (Bruce & Uhlenbeck, 1982). Additional experiments showed that replacement of G20 (in the dihydrouridine loop) or of A73 (in the single-stranded $ACCA_{OH}$ 3′ terminus) each resulted in a 12-fold reduction in catalytic efficiency [see Sampson and Uhlenbeck (1988, 1989)]. Because G20 is present in $tRNA^{Phe}$ but not in any of the other reported yeast tRNA sequences, it could act as an important discriminatory nucleotide.

E. coli $tRNA^{Phe}$ encodes four of the five aforementioned nucleotides, with a U replacing G20. Substitution of G for U20 improves k_{cat}/K_m for the yeast enzyme by 12-fold so that the yeast and *E. coli* substrates are almost equivalent (Sampson & Uhlenbeck, 1989) (Table II). This corresponds to a relatively small amount (1.5 kcal mol^{-1}) of free energy.

Yeast $tRNA^{Arg}$, $tRNA^{Met}$, and $tRNA^{Tyr}$ were reconstructed so that each contained a complete set of the five important nucleotides (Sampson & Uhlenbeck, 1989). Each is converted to a substrate for the homologous yeast phenylalanine tRNA synthetase (Table II). The parameter k_{cat}/K_m for each of the substituted tRNAs is within 50% of that for the cognate yeast $tRNA^{Phe}$. It is not known whether the nucleotide substitutions that were introduced in each case have an effect on the respective cognate enzyme. This information would clarify whether the changes that improve each as a substrate for the phenylalanine enzyme are sufficient to convert the amino acid specificity to phenylalanine only or whether the resultant tRNAs are charged with phenylalanine and one or more additional amino acids. Analytical aminoacylation experiments with other synthetases should make possible a quantitative evaluation of the various competitive effects. Eventually, each mutant tRNA (which charges in vitro with phenylalanine) should be investigated in the context of all of the synthetases and tRNAs in vivo.

Table II: Relative Values of k_{cat}/K_m for Aminoacylation with Phenylalanine of Synthetic tRNAs with Different Subtitutions

starting sequence	substitutions	rel k_{cat}/K_m[a]
yeast $tRNA^{Phe}$	none	1.0
yeast $tRNA^{Phe}$	U20	0.08
E. coli $tRNA^{Phe}$	none	0.04
E. coli $tRNA^{Phe}$	G20	0.5
yeast $tRNA^{Met}$	several[b]	0.7
yeast $tRNA^{Arg}$	several[b]	0.6
yeast $tRNA^{Tyr}$	several[b]	1.5

[a] Data were obtained with yeast phenylalanine tRNA synthetase at pH 7.45 and are taken from Samson et al. (1989). [b] Substitutions were introduced into the "starting sequence" so as to have G20, G34, A35, A36, and A73, which are the nucleotides believed important for the recognition of yeast $tRNA^{Phe}$.

On the basis of an in vivo amber suppression assay, McClain and Foss (1988b) have indicated that more than 5 nucleotides (i.e., 10) are important for the recognition of *E. coli* $tRNA^{Phe}$ by *E. coli* phenylalanine tRNA synthetase. Only two of these (positions 20 and 73) are at positions corresponding to the locations of the five sites studied by Sampson and Uhlenbeck (1989). The relative significance of each of these is unknown. It will be necessary to perform in vitro aminoacylation measurements similar to those done with the yeast enzyme in order to understand more fully the similarities and differences in the recognition by these two enzymes which are specific for the same amino acid.

Modified Bases. Examples Where They Do and Do Not Have an Important Role. Transfer RNAs isolated from natural sources contain several bases at specific locations that are posttranscriptionally modified. Some of these are common to most tRNAs (such as 7-methylguanosine, dihydrouridine, and pseudouridine), and others are unique to a particular amino acid specific tRNA [such as the wybutosine at position 37 of certain eukaryote phenylalanine tRNAs (Sprinzl et al., 1987)]. In the aforementioned studies of *E. coli* $tRNA^{Met}$ and $tRNA^{Val}$ and of yeast $tRNA^{Phe}$, the tRNA substrates were enzymatically synthesized in vitro. As a consequence, these substrates contain no modified bases. When these synthetic substrates were compared with their counterparts as isolated from natural sources (which thus contain the full complement of modified bases), only small differences in aminoacylation kinetics were observed (Sampson & Uhlenbeck, 1988; Schulman & Pelka, 1988). Thus, these are among the examples [see also Samuelsson et al. (1988) and Francklyn and Schimmel (1989)] where the modified bases do not play a major role in recognition by the cognate enzyme in vitro, although they could act to interfere with the interactions of noncognate enzymes.

In contrast, a modification of an isoacceptor of $tRNA^{Ile}$ is essential for recognition by isoleucine tRNA synthetase. In addition to promoting aminoacylation with isoleucine, the modification blocks misacylation by methionine tRNA synthetase. The $tRNA_2^{Ile}$ isoacceptor reads AUA codons. The gene for this isoacceptor encodes a CAT anticodon. Thus,

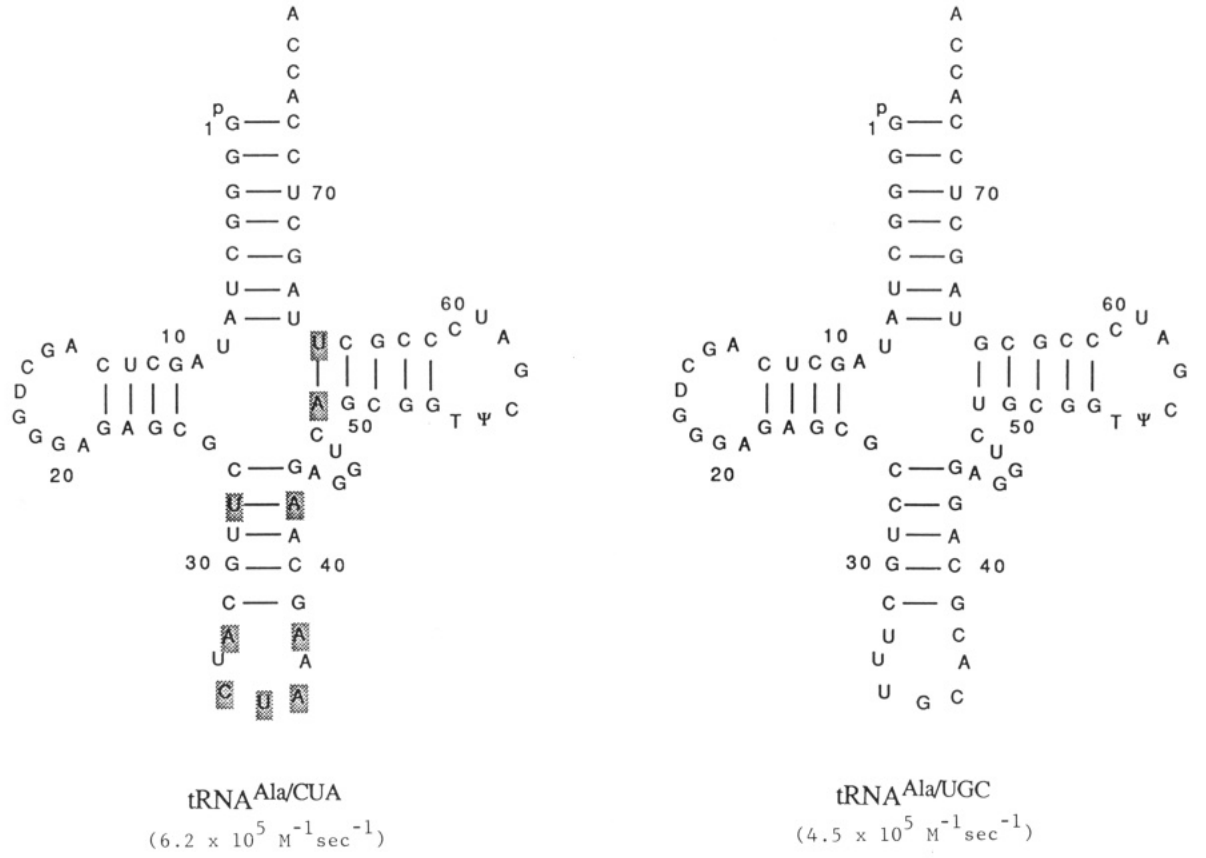

FIGURE 3: Nucleotide sequences and cloverleaf structures of tRNA$^{Ala/UGC}$ and tRNA$^{Ala/CUA}$. The nine nucleotide sequence differences between the natural species (tRNA$^{Ala/UGC}$) and the amber suppressor (tRNA$^{Ala/CUA}$) used in studies of Hou and Schimmel (1988) are shown by shading. Analytical aminoacylation measurements with purified alanine tRNA synthetase have shown that at pH 7.5, 37 °C, there is little difference in the apparent rate (k_{cat}/K_m) of aminoacylation (given in parentheses) of these two tRNA species. Adapted from Park et al. (1989).

without posttranscriptional modification the anticodon is CAU, which is the same as the anticodon for tRNA^{Met}. As shown by Schulman and co-workers, this triplet is recognized by the methionine tRNA synthetase (see Table Ia). Muramatsu et al. (1988a) have found that tRNA$_2^{Ile/CAU}$ is a substrate in vitro for methionine tRNA synthetase. By comparison, however, it is a poor substrate for isoleucine tRNA synthetase. In the mature tRNA, C34 is posttranscriptionally modified to lysidine L (Muramatsu et al., 1988b). This modification consists of a lysine substituted for O-2 of the cytidine ring and attached through the ϵ-amino group directly to C-2 of the pyrimidine base. With this modification, tRNA$_2^{Ile/LAU}$ is efficiently aminoacylated with isoleucine and cannot be aminoacylated with methionine. Thus, the amino acid acceptance in vitro switches according to the state of modification.

The same *E. coli* isoleucyl-tRNA synthetase aminoacylates the major isoacceptor, which has a GAU anticodon. Thus, if the anticodon is the primary site of recognition, then the enzyme recognizes a structural feature common to GAU and LAU.

Effect of a Single Base Pair on the Binding and Catalytic Parameters for the Molecular Recognition of Alanine Transfer RNA

Comparison of Kinetic Behavior of an Alanine-Inserting Amber Suppressor with a Naturally Occurring Isoacceptor. The alanine system has been used to explore the effects on tRNA binding and catalytic parameters of single nucleotide substitutions, which alter the G·U base pair, and thereby obtain greater insight into the molecular basis for discrimination of a simple structural feature. The tRNA$^{Ala/CUA}$ amber suppressor described above was based on the sequence of the tRNA$^{Ala/GGC}$ isoacceptor. The second known naturally occurring isoacceptor is tRNA$^{Ala/UGC}$. There are nine nucleotide differences between the synthetic tRNA$^{Ala/CUA}$ amber suppressor and tRNA$^{Ala/UGC}$. Eight of these are located in the anticodon stem and loop, and the ninth is a position 49·65 base pair in the TUC stem (Figure 3). The kinetic parameters for these two tRNAs at pH 7.5, 37 °C, are $k_{cat} = 1.0\ s^{-1}$ and $K_m = 2.2\ \mu M$ for tRNA$^{Ala/UGC}$ and $k_{cat} = 1.8\ s^{-1}$ and $K_m = 2.9\ \mu M$ for tRNA$^{Ala/CUA}$ (Park et al., 1989). The small differences between the respective parameters may be experimentally insignificant, and the apparent second-order rate constants k_{cat}/K_m are close in value (Figure 3).

The binding of the two tRNA species has been measured at pH 5.5, where the nitrocellulose filter assay has a high efficiency for retention of synthetase–tRNA complexes. [Above pH 6.0 the efficiency drops so that the method cannot be used to measure binding (Yarus & Berg, 1970). In general, association of tRNAs with aminoacyl-tRNA synthetases increases at lower pH values (Schimmel & Soll, 1979).] At pH 5.5, 23 °C, the dissociation constants for the enzyme–tRNA complexes are within experimental error of each other (Park et al., 1989). Thus, by kinetic and equilibrium measurements, the nine nucleotide differences between tRNA$^{Ala/CUA}$ and tRNA$^{Ala/UGC}$ have little or no effect on binding and catalytic parameters. This is consistent with genetic studies which show that substitutions in the anticodon loop and stem, and of the position 49·65 base pair in the TUC stem, do not interfere with acceptance of alanine (Hou & Schimmel, 1988). It is of interest to note that the anticodon of yeast tRNA^{Ala} is also not required for recognition by the yeast enzyme (Jin et al., 1987).

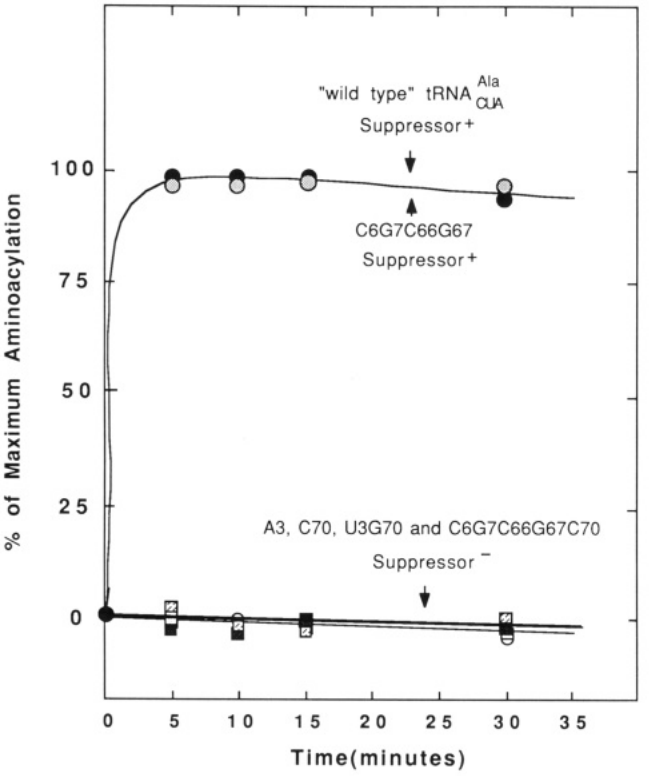

FIGURE 4: Substrate levels of alanine tRNA synthetase do not significantly aminoacylate A3·U70, G3·C70, or U3·G70 $tRNA^{Ala}$ at pH 7.5, 37 °C. For these experiments, the enzyme and tRNA concentrations were 20 and 4 μM, respectively. The suppressor$^+$ or suppressor$^-$ designation indicates which tRNAs are able to suppress the *trpA(UAG234)* amber allele in vivo. Note also that the C6G7C66G67 $tRNA^{Ala/CUA}$ has four substitutions in the acceptor helix and these do not prevent aminoacylation with alanine. Adapted from Park et al. (1989).

Effect of Sequence Variants of the G3·U70 Base Pair on Kinetic Parameters. The initial characterization of sequence variants of the G3·U70 base pair showed that they were defective for aminoacylation in vitro, but it was not determined whether the defect is in k_{cat}, K_m, or both (Hou & Schimmel, 1988). In further experiments, it was shown that addition of excess A3·U70 $tRNA^{Ala/CUA}$ does not inhibit aminoacylation of $tRNA^{Ala/CUA}$ at pH 7.5, 37 °C. The experiments were done under conditions where an inhibition constant of less than 95 μM would have been detected. Because the K_m for $tRNA^{Ala/CUA}$ is 2.2 μM, and because of evidence that the K_m can be regarded as an enzyme–tRNA dissociation constant, the result implies that at pH 7.5 (37 °C) the binding constant for the A3·U70 variant is at least 40-fold weaker than that for the natural $tRNA^{Ala/UGC}$ isoacceptor (Park et al., 1989).

This is not the only effect of the A3·U70 substitution, however. An excess of enzyme over tRNA substrate has been used to attempt aminoacylation of A3·U70, G3·C70, U3·G70, and C6C7G66G67C70 $tRNA^{Ala/CUA}$. (The latter species has five substitutions in the acceptor stem, which collectively change three base pairs, including the one at position 3·70.) Because of the excess of enzyme, it does not have to turn over in order to achieve complete aminoacylation. With 20 μM enzyme and 4 μM tRNA substrate, the "wild-type" $tRNA^{Ala/CUA}$ and C6C7G66G67 $tRNA^{Ala/CUA}$ species are immediately aminoacylated, as expected. Even after a 30-min incubation, however, the position 3·70 variants are not aminoacylated (Figure 4). If the position 3·70 variants could be aminoacylated but the kinetic defect caused an extremely low product release, then "one shot" of aminoacylation would have been detected at these high enzyme concentrations. Thus, the defect is at a step prior to stable aminoacyl-tRNA formation.

The failure to observe aminoacylation of position 3·70 variants after prolonged incubations with excess enzyme at pH 7.5 suggests that, in addition to reduced binding, there could be a severely reduced k_{cat}. This was demonstrated in the following way. At pH 5.5, the binding of synthetases to tRNAs is generally stronger and can be independently measured by the nitrocellulose filter assay. Thus, experiments were attempted at pH 5.5 to determine whether, under these conditions, the binding of the A3·U70 variant might be enhanced so that a complex could be demonstrated and tested for catalytic competence. The rate of aminoacylation of the wild-type $tRNA^{Ala}$ is reduced at pH 5.5, but the K_d measured by the filter assay for the enzyme–$tRNA^{Ala}$ complex (0.28 μM) is in agreement with the K_m measured in the aminoacylation assay under the same conditions (0.22 μM). Binding of A3·U70 $tRNA^{Ala}$ can be detected at pH 5.5 by the nitrocellulose filter assay, and the K_d of 1.2 μM is only 4-fold less than that of the wild-type $tRNA^{Ala}$. However, no aminoacylation of the A3·U70 species can be detected, even though binding was demonstrated. Moreover, the A3·U70 variant is a competitive inhibitor of aminoacylation of wild-type $tRNA^{Ala}$ at pH 5.5. The inhibition constant K_I = 1.5 μM is close to the independently measured K_d. Because the A3·U70 species binds competitively, it is likely that it occupies the same site on the enzyme as wild-type $tRNA^{Ala}$ (Park et al., 1989).

These data show that, even when bound to alanine tRNA synthetase, A3·U70 $tRNA^{Ala}$ cannot be aminoacylated in vitro. The discrimination against this species is a double barrier of both k_{cat} and K_m parameters. Thus, there is a severe reduction in k_{cat}/K_m for the aminoacylation with alanine of tRNAs that have A3·U70 or G3·C70 or other alternatives at position 3·70 (cf. Figure 4). Because the presence of the G3·U70 pair in a number of tRNA sequence frameworks is sufficient to confer aminoacylation with alanine, the enzyme had to develop a rigorous way to distinguish $tRNA^{Ala}$ from those tRNAs that differ by only a single nucleotide at position 3·70. These tRNAs include those for glutamine, glycine, histidine, leucine, lysine, tryptophan, and valine, which have a G3·C70 base pair, and specific isoacceptors of arginine and serine tRNAs, which have an A3·U70 base pair (Sprinzl et al., 1987). The sharply reduced binding at pH 7.5 of those species that differ by only one nucleotide at the 3·70 position, as demonstrated for the A3·U70 variant, also prevents them from being inhibitors of the enzyme under these conditions.

Evidence for Interaction of Alanine Transfer RNA Synthetase with the Amino Acid Acceptor Helix

RNA Footprinting. A three-dimensional structure of alanine tRNA synthetase or of the synthetase–tRNA complex is not available, although diffraction-grade crystals of a fragment of the enzyme have recently been reported (Frederick et al., 1988). In the absence of high-resolution structural information, RNA "footprint" methods can provide a rough model of sites on $tRNA^{Ala}$ that make contact with the bound enzyme. Nuclease digestion of the free and bound tRNA is done under conditions where approximately one cut per molecule is introduced. When end-labeled tRNA is used and the digested species are resolved by gel electrophoresis, the positions of the cleavages can be accurately determined. Because nucleases preferentially cleave at some sites and far less at others, not every position in the molecule can be investigated by these methods.

Figure 5a shows the sequence and cloverleaf structure of $tRNA^{Ala/UGC}$ and indicates by arrows the 32 phosphodiester

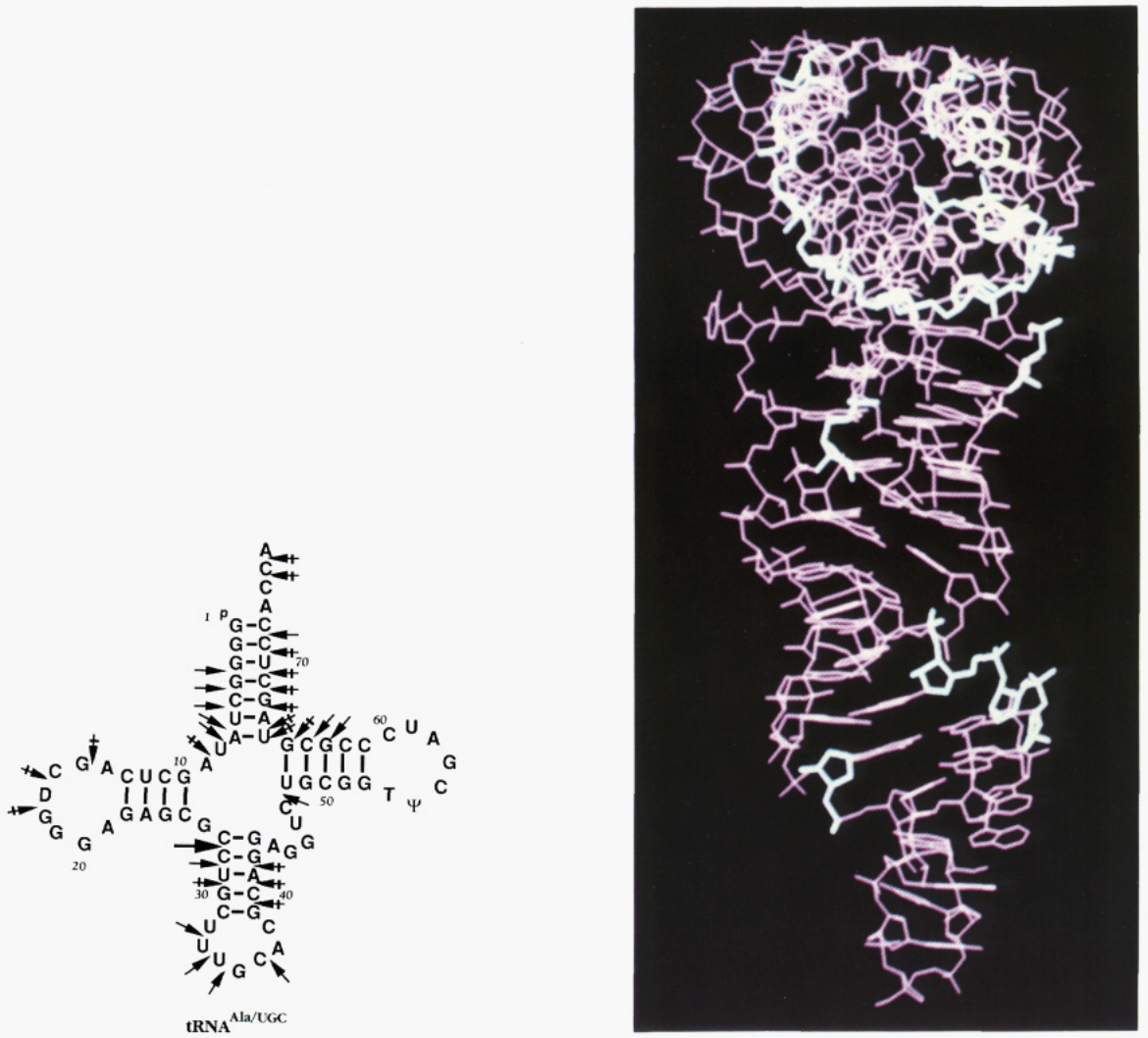

FIGURE 5: (a, left) Sequence and cloverleaf structure of tRNA$^{Ala/UGC}$ and the phosphodiester linkages that are protected from ribonuclease attack by bound alanine tRNA synthetase. The arrows designate the positions that have been examined by use of RNase A or RNase V_1. The arrows with slashed bars are the sites protected by the bound synthetase. The heavy arrow denotes a site of enhanced cleavage in the presence of bound enzyme. Adapted from Park and Schimmel (1988). (b, right) Skeletal model of tRNA^{Ala} as viewed from the 3′ end with sites protected from nuclease cleavage by bound alanine tRNA synthetase. The backbone has been highlighted in white at the places that are protected by the bound enzyme. The protection of the 3′ side of the acceptor helix [see (a)] is seen as a spiral that follows the course of the acceptor–TΨC helix and encompasses the G3·U70 base pair.

linkages that have been probed by the nuclease digestion assay (Park & Schimmel, 1988). For this experiment, RNase A (specially cleaves on the 3′ side of pyrimidines with a preference for single-stranded regions) and cobra venom RNase V_1 (preferentially cleaves on the 5′ side of a purine or pyrimidine base in a double-stranded region) were used. The arrows with cross bars denote those sites that are protected from cleavage by the bound enzyme. The anticodon loop is not protected, and this is consistent with genetic and in vitro kinetic results which show that the anticodon is not important for recognition. However, there is protection (and a site of synthetase-induced hypersensitivity) in the anticodon stem, even though genetic studies have suggested that sequence alterations in this region do not disrupt aminoacylation with alanine in vivo. This simply shows that regions of protection are not necessarily of major consequence for determining amino acid specificity. The greatest concentration of consecutive protected sites is on the 3′ side of the amino acid acceptor helix and extends into the TΨC helix. [These two helices are fused together as one helical branch of the L-shaped tRNA structure (Figure 2).] Both phosphodiester bonds that flank U70 are shielded by the bound enzymes. There is no protection of the 5′ side of the acceptor helix, although the internucleotide phosphodiester linkage between U8 and A9 is protected.

The pattern of protection of the acceptor helix implies that the enzyme spirals around the acceptor–TΨC helix. This spiral is evident by viewing the structure "on end", looking from the 3′ end down the axis of the helix (Figure 5b). The G3·U70 base pair is visible in this projection. If the enzyme recognizes specifically this base pair, and if this pair is arranged in the wobble configuration, then it is formally possible to distinguish G3·U70 from other base pairs. In the wobble configuration, the 4-keto oxygen of U70 is not hydrogen bonded and it projects into the major groove. Introduction of A70 introduces a standard Watson–Crick A·U base pair whereby the 4-keto of uracil is now shifted into a hydrogen bond. On the other hand, change of U70 to C70 introduces an exocyclic amino group at the 4-position which is hydrogen bonded in the standard Watson–Crick configuration with the 6-keto group of guanine. Similar considerations show that the exocyclic 2-amino group of guanine, which is not hydrogen bonded in a G3·U70 wobble pair, can be a site for discrimination. These explanations of specificity are speculative, although they demonstrate how discrimination of subtle structural alterations is possible in principle.

Table III: Apparent Kinetic Parameters for Aminoacylation of $tRNA^{Ala}$ and of Mini- and Microhelices[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
$tRNA^{Ala}$	2.0	0.89	4.4×10^5
$minihelix^{Ala}$	11.4	1.5	1.3×10^5
G3·U70 $minihelix^{Tyr}$	8.8	0.48	5.5×10^4
$microhelix^{Ala}$	35.9	0.28	7.8×10^3

[a] Adapted from Francklyn and Schimmel (1989). Results were obtained at pH 7.5, 37 °C, at a saturating concentration of ATP and a subsaturating concentration (20 μM) of radioactive alanine. Because of the high K_m for alanine, saturation requires prohibitively large amounts of radioactive substrate. Earlier work showed that the K_m for $tRNA^{Ala}$ exhibited little sensitivity to the concentration of alanine (Jasin & Schimmel, 1985).

Recognition of the G3·U70 Base Pair and/or of a Structural Variation? Recognition of $tRNA^{Ala}$ may also be directed, at least in part, at a structural variation in the amino acid acceptor helix that arises from the G3·U70 base pair. McClain et al. (1988) report that other nucleotide combinations including U3·G70 and A3·U70, while weak or inactive suppressors of the alanine-requiring *trpA(UAG234)* amber allele, can nonetheless insert alanine at an amber codon of dihydrofolate reductase. The interpretation of this result is unclear because of the poor or nonexistent suppression of the *trpA(UAG234)* amber by these $tRNA^{Ala}$ sequence variants and because of the inability to detect significant aminoacylation of the A3·U70 or U70·G3 $tRNA^{Ala}$ species in vitro with substrate levels of enzyme (Figure 4). Possibly the enzyme has a residual activity toward tRNA substrates that have structural irregularities in the acceptor helix and that, in vivo, a minor amount of the aminoacylated A3·U70 and U3·G70 species is produced and is then sequestered by elongation factor Tu and carried to the ribosomes.

Aminoacylation with Alanine of RNA Minihelices

Because of the aforementioned evidence that a major portion of the alanine tRNA synthetase–$tRNA^{Ala}$ interaction is concentrated in the acceptor–TΨC helix, RNA hairpin helices have been designed and synthesized to correspond to this part of the molecule (Francklyn & Schimmel, 1989). These minihelices have been tested as substrates for aminoacylation with alanine tRNA synthetase. In $minihelix^{Ala}$ the acceptor–TΨC helix based on $tRNA^{Ala/GGC}$ has been recreated (Figure 6). This has 12 base pairs with a loop of seven nucleotides and a single-stranded 3′ end that terminates in the sequence $ACCA_{OH}$. This construction effectively deletes the segment from A9 to C48 of $tRNA^{Ala/GGC}$ so that the highly conserved nucleotide U8 has been covalently joined to A49. A G3·C70 $minihelix^{Ala}$ variant has also been constructed.

The wild-type $minihelix^{Ala}$ is efficiently and completely aminoacylated by alanine tRNA synthetase. When compared to $tRNA^{Ala}$, the k_{cat} parameter is similar and K_m is about 6-fold higher for the minihelix (Table III). The elevation in the K_m corresponds to approximately 1 kcal mol^{-1}. This is a small energy and could be due to one or two van der Waals contacts that are missing in the enzyme–$minihelix^{Ala}$ complex.

A G3·C70 variant of $minihelix^{Ala}$ was also synthesized and was found to be inactive for aminoacylation with alanine, even with elevated levels of alanine tRNA synthetase. This behavior is analogous to the effect of G3·U70 in the aminoacylation of $tRNA^{Ala}$. To determine whether the G3·U70 base pair could confer alanine acceptance on an unrelated minihelix, a "tyrosine" minihelix with a G3·U70 variant was synthesized. The $minihelix^{Tyr}$ is based on the acceptor–TΨC sequence of $tRNA^{Tyr}$ and differs at 7 of the 12 base pairs (in the helical region) from that of $minihelix^{Ala}$. The presence of the G3·U70 base pair is required for the efficient aminoacylation of this hairpin. Relative to $minihelix^{Ala}$, K_m for G3·U70 $minihelix^{Tyr}$ is comparable and k_{cat} is reduced about 3-fold (Table III). In terms of energy, the reduction in k_{cat} is small and corresponds to less than 1 kcal mol^{-1} (Francklyn & Schimmel, 1989).

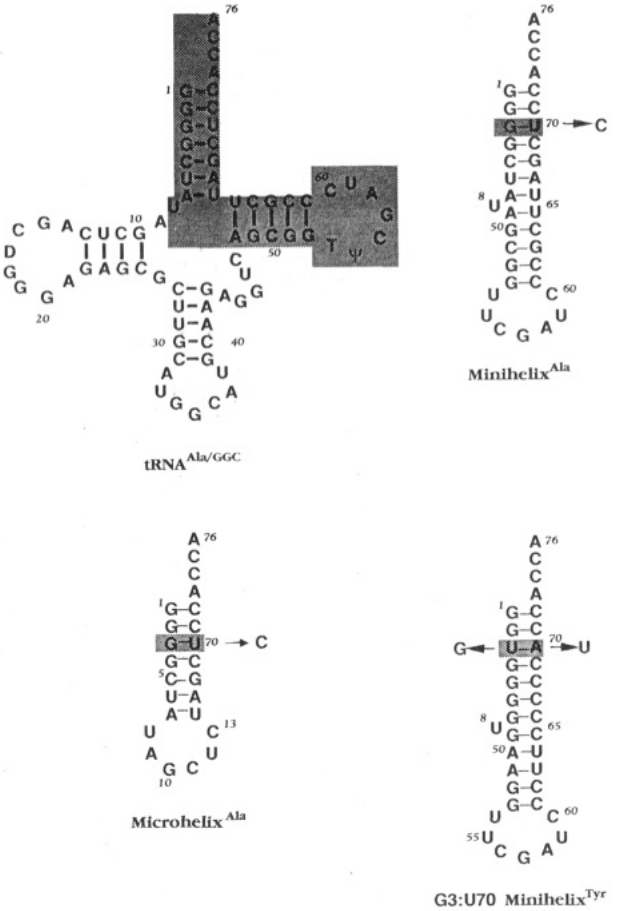

FIGURE 6: RNA minihelices and a microhelix that can be aminoacylated by alanine tRNA synthetase. The alanine helices are based on the sequence of $tRNA^{Ala/GGC}$. $Minihelix^{Ala}$ includes the acceptor–TΨC helix and joins U8 to A49, while $microhelix^{Ala}$ consists only of the acceptor helix (seven base pairs) and joins C13 to U66. In each case, substitution of C70 for U70 eliminates efficient aminoacylation with alanine. The tyrosine minihelix ($minihelix^{Tyr}$) is based on the acceptor–TΨC helix of $tRNA^{Tyr}$; this minihelix becomes an efficient substrate for alanine tRNA synthetase when G3·U70 is substituted for U3·A70 (Francklyn & Schimmel, 1989).

Encouraged by the success of these experiments, an even smaller synthetic helix was constructed, which is based on just the amino acid acceptor stem. This consists of seven base pairs connected by a loop of six nucleotides (Figure 6). In this $microhelix^{Ala}$, the sequence of the loop starts at U8 and extends to C13 which is joined to U66. The $microhelix^{Ala}$ is completely aminoacylated by alanine tRNA synthetase at a rate which is reduced relative to $tRNA^{Ala}$. While the K_m is 18-fold higher for $microhelix^{Ala}$, the k_{cat} is reduced by only 3-fold (Table III). On the basis of the relative K_m parameters, the decrease in binding energy is about 1.7 kcal mol^{-1}. The relatively small effect on k_{cat} shows that, once bound to the enzyme, the rate of aminoacylation of the microhelix is comparable to that of intact $tRNA^{Ala}$.

Because the seven base pair $microhelix^{Ala}$ has a k_{cat} for aminoacylation that is within a factor of 3 of that of $tRNA^{Ala}$ (Table III), it is evident that this parameter is not greatly enhanced by sequences which lie outside of the acceptor helix. Moreover, most of the binding energy is derived from interactions with the acceptor–TΨC helix. However, because

$tRNA^{Ala}$ must be discriminated in vivo from the ensemble of noncognate tRNAs, it is possible that there are negative determinants outside of the acceptor–TΨC helix which prevent $tRNA^{Ala}$ from fitting into the tRNA binding sites of other synthetases. In the mapping of contacts between $tRNA^{Ala}$ and its cognate enzyme (Figure 5), sites are protected that are not present in the minihelices which are efficient substrates for the enzyme (Figure 6 and Table III). These could be regions where tRNAs other than $tRNA^{Ala}$ have nucleotides which block their binding to alanine tRNA synthetase and thereby lessen the likelihood that they will inhibit the enzyme.

It has been speculated that, at an early stage in the development of an organized system for protein synthesis, small oligonucleotides may have been aminoacylated and that sequence information in those oligonucleotides could have conferred some specificity for amino acid attachment. The results described above show that, for at least one amino acid, an oligonucleotide can be enzymatically aminoacylated. The proximal location of the enzyme's recognition site relative to the amino acid attachment site was critical to the success of these experiments. It is doubtful that mini- or microhelices analogous to those for alanine (Figure 6) could be aminoacylated in those cases where the major determinants for identity are located in the anticodon or another region (see below) which is distal to the amino acid acceptor end. In those instances it is conceivable that a determinant for identity was at one time proximal to the acceptor end of a small oligonucleotide and, as the tRNA structure became fully elaborated, was translocated to a distal position. Regardless of the origin of the structure of tRNA and of the basis for specific amino acid attachment, however, further investigation of oligonucleotide components of tRNAs will afford a deeper analytical understanding of recognition.

Concluding Remarks

Positive and Negative Determinants for Recognition, the Role of Competition, and the Difficulties of Interpretation. Conceptually, it is necessary to distinguish nucleotides that are recognized by an aminoacyl-tRNA synthetase from those that block or interfere with binding or catalysis. These can be considered as positive and negative determinants, respectively. It is possible for the context of a particular tRNA sequence to inhibit the recognition of an element which is important for identity; that is, a negative determinant may be dominant. For this reason it is necessary to test nucleotides believed important for recognition in more than one sequence framework. A clear example of context effects is shown in the different degree of aminoacylation with alanine in vivo when the G3·U70 base pair is transferred into $tRNA^{Cys/CUA}$ versus into $tRNA^{Phe/CUA}$ (Hou & Schimmel, 1988). In addition, the relative levels of aminoacyl-tRNA synthetases and tRNAs can play a role in determining the recognition of a particular tRNA and whether a given mutant species will be recognized [Yarus et al., 1986; Swanson et al., 1988; see also Yarus (1988)].

There is a clear conceptual distinction between effects due to negative elements and those due to positive elements, and in some cases further experiments need to be done in order to make this distinction. Thus, nucleotide substitutions that allow $tRNA^{Tyr/CUA}$ (Figure 1a) and $tRNA^{Ser/CUA}$ (Rogers & Soll, 1988) to be aminoacylated with glutamine may be effective by blocking the interaction of the mutant tRNAs with the cognate enzyme (tyrosine and serine tRNA synthetases, respectively) rather than by creating a site for the interaction with glutamine tRNA synthetase [that is, in addition to the middle base (U) of the anticodon which is present in the amber suppressor]. Similarly, mutations in the anticodon stem loop of an *E. coli* $tRNA^{Gln/CUA}$ amber suppressor which allow some aminoacylation with tryptophan may weaken the interaction with glutamine tRNA synthetase rather than create specific sites for recognition by tryptophan tRNA synthetase, particularly because some of these substitutions do not recreate nucleotides that are in the analogous locations of $tRNA^{Trp}$ (Yamao et al., 1988a,b). If the nucleotides are acting as blocking elements for glutamine tRNA synthetase, then their transfer into another tRNA sequence framework is not likely to confer tryptophan acceptance.

Limited Nucleotide Constellations Establish the Identities of Some Transfer RNAs. The standards of the field have been raised so that it is no longer sufficient to show only that a specific nucleotide substitution lowers the efficiency of aminoacylation with a particular enzyme. This is because some substitutions that interfere with aminoacylation could create new, unfavorable enzyme–tRNA steric conflicts at sites which are close to but which do not bind to the enzyme through specific atomic interactions in the wild-type complex. The more recent conclusions are based upon the now accepted practice of making nucleotide substitutions into different tRNA sequence frameworks and establishing the effect on amino acid acceptance. This experiment attempts to identify sites that are dominant positive determinants in vivo and in vitro (thorough k_{cat} and/or K_m parameters).

The locations of important nucleotides for some tRNAs are given in Table IV. The listing includes cases where a "transfer" experiment has been done and is provisional because further work may show that, for a particular tRNA, additional nucleotides are required. Moreover, context effects undoubtedly have a major role (see above), and the full extent of these effects will not be known for some time. However, it is now clear that the determinants for identity are idiosyncratic. Also, for at least some tRNAs, a limited constellation of nucleotides is a major determinant of their identities. It is noteworthy that there is evidence that a simple structural feature (base-pair mismatch at the first position of the amino acid acceptor helix) is a major determinant for distinguishing an initiator from an elongator tRNA (Seong & RajBhandary, 1987).

Possible Role for Editing in the Determination of Transfer RNA Identity. An early observation by Baldwin and Berg (1966) indicated that valine could be activated by isoleucine tRNA synthetase and that $tRNA^{Ile}$ then induced hydrolysis of the bound valyl adenylate. There was no evidence that valine was actually transferred to $tRNA^{Ile}$. Schreier and Schimmel (1972) then showed that aminoacyl-tRNA synthetases have a hydrolytic site that removes an amino acid from transfer RNA, in the absence of AMP and pyrophosphate. This was initially discovered as an activity which cleaves the ester bond which links the cognate amino acid to its tRNA, and evidence was presented that the activity is general to synthetases

$$\text{Ile-tRNA}^{Ile} + \text{Ile-tRNA synthetase} \rightarrow \text{Ile} + \text{tRNA}^{Ile} + \text{Ile-tRNA synthetase}$$

$$\text{Phe-tRNA}^{Phe} + \text{Phe-tRNA synthetase} \rightarrow \text{Phe} + \text{tRNA}^{Phe} + \text{Phe-tRNA synthetase}$$

and, similarly, for other aminoacyl-tRNA synthetases.

Subsequently, Eldred and Schimmel (1972) showed that, for isoleucine tRNA synthetase, this activity is much enhanced when the incorrect amino acid is attached to a tRNA:

$$\text{Val-tRNA}^{Ile} + \text{Ile-tRNA synthetase} \rightarrow \text{Val} + \text{tRNA}^{Ile} + \text{Ile-tRNA synthetase}$$

Table IV: Nucleotides Implicated as Important for the Identities of Some Transfer RNAs

tRNA	important positions	evidence
E. coli alanine	G3·U70	in vivo amber suppression with over 30 $tRNA^{Ala}$ sequence variants and with G3·U70 $tRNA^{Cys/CUA}$ and G3·U70 $tRNA^{Phe/CUA}$; in vitro aminoacylation data on several $tRNA^{Ala}$ variants on synthetic minihelix and microhelix substrates [Hou & Schimmel, 1988; Park et al., 1989; Francklyn & Schimmel, 1989; cf. also McClain and Foss (1988a)]
E. coli arginine	A20 and others	preliminary results in vivo based on introducing A20 and A59 into a $tRNA^{Phe}$ amber suppressor; A59 may play a structural role rather than a role in direct recognition; other nucleotides such as those in anticodon may also be important (McClain & Foss, 1988c)
E. coli glutamine	U35	some tRNAs with the amber codon are misacylated with glutamine, and U35 appears responsible (see text); other nucleotides in the acceptor helix may be important as well
E. coli isoleucine	L34	posttranscriptional modification of cytidine 34 to lysidine switches $tRNA^{Ile}_2$ from a methionine-accepting to an isoleucine-accepting tRNA in vitro (Muramatsu et al., 1988a)
E. coli methionine	anticodon	in vitro aminoacylation data with anticodon sequence variants and transfer of the CAU anticodon into $tRNA^{Val}$ (Schulman & Pelka, 1988)
yeast phenylalanine	G20, G34, A35, A36, A73	in vitro aminoacylation data with sequence variants of $tRNA^{Phe}$ and of four reconstructed tRNAs (Samson et al., 1989)
E. coli serine	G1·C72, G2·C71, A3·U70, C11·G24	in vivo amber suppression with sequence variants of a $tRNA^{Leu}$ that was converted to a serine-accepting tRNA [Normanly et al., 1986a; cf. Schulman and Abelson (1988)]
E. coli valine	anticodon	in vitro aminoacylation data with the UAC anticodon of $tRNA^{Val}$ transferred into $tRNA^{Met}$ (Schulman & Pelka, 1988)

and Yarus (1972) demonstrated a rapid deacylation of Ile-$tRNA^{Phe}$ by phenylalanine tRNA synthetase:

$$\text{Ile-tRNA}^{\text{Phe}} + \text{Phe-tRNA synthetase} \rightarrow \text{Ile} + \text{tRNA}^{\text{Phe}} + \text{Phe-tRNA synthetase}$$

Considerable investigation of editing reactions was subsequently undertaken [see summaries by Soll and Schimmel (1974), Schimmel and Soll (1979), Yarus (1979), and Fersht (1985)]. Editing can occur by hydrolysis of the aminoacyl adenylate or by charging followed by hydrolysis of the mischarged aminoacyl-tRNA species. The editing activity of a specific enzyme is generally directed toward an amino acid whose steric bulk is not greater than that of the cognate amino acid. Such amino acids can potentially be activated by a specific enzyme (e.g., valine can be activated by isoleucine tRNA synthetase and threonine by the valine enzyme) because they "fit" into the amino acid binding site, albeit with a lower affinity.

The role of editing in vivo is affected by the presence of elongation factor Tu, which tightly binds and sesquesters aminoacyl-tRNA species (Schimmel & Soll, 1979). Thus, if a mischarged tRNA species is released from an enzyme, it can be carried into the ribosomal translation apparatus by Tu and insert a missense substitution in a growing polypeptide chain. The role of editing of charged mutant amber suppressors in vivo, and the influence of this potential reaction on the results obtained with the amber suppression assay, is unknown. When a G3·U70 base pair is transferred into $tRNA^{Cys/CUA}$, the resulting mutant amber suppressor can be aminoacylated (with alanine) in vitro with purified alanine tRNA synthetase (Hou & Schimmel, 1988). The overall yield of aminoacylation of Ala-G3·U70 $tRNA^{Cys/CUA}$ is dependent on the enzyme concentration, however, and may reflect an editing reaction of the alanine tRNA synthetase that is due to enzyme-catalyzed deacylation of Ala-G3·U70 $tRNA^{Cys/CUA}$ and, additionally or alternatively, to some hydrolysis of the enzyme-bound alanyl adenylate when presented with G3·U70 $tRNA^{Cys/CUA}$. Specific editing reactions with "transformed" tRNAs remain to be demonstrated, but these considerations illustrate the role that editing may have in determining the identity of transfer RNAs.

Defining Sites on Aminoacyl-tRNA Synthetases That Determine Transfer RNA Identity. Structural information on aminoacyl-tRNA synthetases has been limited to *Bacillus stearothermophilus* tyrosine and *E. coli* methionine tRNA synthetases (Bhat et al., 1982; Risler et al., 1982; Blow et al., 1983; Brunie et al., 1987). A cocrystal with tRNA has not been obtained in either case. For tyrosine tRNA synthetase, there is a nucleotide fold in the amino-terminal half which is the location of ATP and tyrosine binding sites which have been extensively analyzed by Fersht and co-workers by site-directed mutagenesis (Fersht et al., 1984; Fersht, 1985). The C-terminal half of the protein is required for binding of $tRNA^{Tyr}$ but is disordered in the crystal (Blow et al., 1982; Bedouelle & Winter, 1986). The tRNA binding site has not been definitively located in the structure of methionine tRNA synthetase, although the tRNA cross-linking experiments of Schulman and co-workers have provided evidence that at least a part of the interaction involves the C-terminal half of the protein (Valenzuela et al., 1984; Valenzuela & Schulman, 1986).

Cocrystals of yeast aspartyl-tRNA synthetase with $tRNA^{Asp}$ (Lorber et al., 1983; Podjarny et al., 1987) and of *E. coli* glutamine tRNA synthetase with $tRNA^{Gln}$ (Perona et al., 1988) have been obtained. The latter crystals diffract to high resolution and will provide the first well-defined structure of a complex. The specific binding interactions in the synthetase–tRNA complexes are relatively weak, and the ones most critical will have to be differentiated from weak nonspecific protein–tRNA contacts. It should be possible from the model of a complex to define nucleotide replacements in the bound tRNA that would not be accommodated in the complex. Such nucleotides may constitute the "negative determinants" described above. The possibility of defining the structural basis for discrimination by the k_{cat} parameter is more problematic, because this is influenced by interactions in a transition state and not in the form which is isolated in a cocrystal. The structural interpretation of mutants of glutamine tRNA synthetase that cause misacylation may be instructive (Inokuchi et al., 1984).

For alanine tRNA synthetase, much of the recognition is concentrated in the amino acid acceptor helix and is centered on the G3·U70 base pair. It is not known whether the enzyme recognizes specific atoms in the base pair or a helix variation at this location. It is noteworthy that the U3·G70 $tRNA^{Ala/CUA}$ is not aminoacylated in vitro by substrate levels of the enzyme (Figure 3). In experiments with nucleases that have respectively a strong preference for cleavage in single- or double-stranded regions, the G3·U70 base pair behaves as though it is part of a double-stranded section (Park & Schimmel, 1988).

Alanine tRNA synthetase is a tetramer of identical 875 amino acid polypeptides (Putney et al., 1981a,b). Eighteen

fragments of the enzyme have been created and analyzed, and from these studies the locations have been defined for regions important for synthesis of alanyl adenylate, reaction of the adenylate with bound tRNA, binding of tRNA, and tetramer formation (Jasin et al., 1983, 1984, 1985; Regan et al., 1987; Hill & Schimmel, 1989). The first 368 amino acids encode a domain that has the adenylate synthesis activity. Sequences in the 93 amino acid segment from Thr369 to Asp461 have a major influence on both k_{cat} and K_m parameters for the tRNA-dependent step of aminoacylation (Jasin et al., 1983, 1984; Ho et al., 1985; Regan et al., 1987, 1988). Because both parameters are sensitive to the presence of a G3·U70 base pair, the region from Thr369 to Asp461 may encode the structure which recognizes the amino acid acceptor helix of $tRNA^{Ala}$. The isolation of mutations in the enzyme that compensate for substitutions at position 3·70 may further define the location of the critical amino acids and may help to interpret the structural information that is being obtained on this enzyme. The conformational analysis and tRNA interactions of synthetic peptides that recreate at least a part of the binding site may also be instructive.

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