E.coli tRNA Leucine Identity and Recognition Sets

Thesis by
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NULLA DIES SINE LINEA

Alla mia famiglia

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ABSTRACT

E. coli contains five different tRNAs which recognize the six leucine codons. These tRNAs are all recognized by the single leucyl-tRNA synthetase (LeuRS). We have used *in vitro* and *in vivo* methods to determine the set of identity elements which distinguish the set of leucine tRNAs from all other tRNAs allowing the faithful translation of the leucine codons.

An identity swap experiment has been used to determine which of the nucleotides conserved in all leucine tRNAs are identity elements. In this experiment the identity of an amber suppressor $tRNA^{Ser}$ was changed completely to leucine. This experiment was effective because the anticodons in $tRNA^{Ser}$ and $tRNA^{Leu}$ are not recognized by their respective synthetases and consequently in both cases tRNAs containing the CUA anticodon required in amber suppressors are fully active.

In its minimal form the Ser-Leu swap required six changes, five of which altered the tertiary structure of the tRNA: the G15-C48 tertiary "Levitt base-pair" in $tRNA^{Ser}$ was changed to A15-U48 found in all leucine tRNAs; it was necessary to insert one nucleotide and to delete one nucleotide so as to position the conserved D-loop G18, G19 nucleotides as they are in all leucine tRNAs; a base was inserted at position 47n between the base-paired extra stem and the T-stem to achieve a configuration found in all leucine tRNAs; in addition it was necessary to change the G73 "discriminator" base in $tRNA^{Ser}$ to A73, found in all leucine tRNAs. This

minimally altered $tRNA^{Ser}$ inserted exclusively leucine as an amber suppressor and it was an excellent *in vitro* substrate for LeuRS.

Both $tRNA^{Ser}$ and $tRNA^{Leu}$ are type II tRNAs containing large base-paired extra stem loops. In the case of $tRNA^{Ser}$ the extra stem loop is a crucial identity element but for $tRNA^{Leu}$ earlier *in vitro* and *in vivo* experiments had indicated that it is not an identity element. To investigate the role of tRNA tertiary structure in leucine identity we carried out a parallel swap experiment in which the glutamine identity of the amber suppressor $tRNA^{Ser\Delta}$ (in which the type II extra stem loop had been replaced by a consensus type I loop) was converted to leucine. This "type I" swap experiment was also successful both *in vivo* and *in vitro*. Interesting differences in the role of conserved leucine base-pairs in the acceptor stems of leucine tRNAs were observed in the two experiments. In the type II swap the conserved acceptor stem bases were not important. In the type I swap their absence had a large effect both *in vivo* and *in vitro*. This result indicates that the presence of the extra stem loop in leucine tRNAs has an effect on the tertiary structure of the tRNA. When this structure is altered conserved nucleotides, unimportant in its presence, take on an important role. Possible reasons for this effect are discussed.

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The Specificity Problem in Protein Synthesis

Introduction

The specific interactions between RNA and protein play a critical role in many cellular processes (1). One of the most interesting and important of these is the specific interaction between aminoacyl RNA synthetases (aaRS) and tRNA. The aaRS must recognize its cognate tRNA, and this interaction determines which amino acid is activated for protein synthesis. Once the tRNA is charged with an amino acid, the fate of that amino acid in protein synthesis is determined by anticodon-codon interactions in the ribosome. So an error in the aaRS recognition step results in the incorporation of the wrong amino acid (2).

The performance required of an aaRS is a difficult one. All tRNAs are similar at the level of primary, secondary, and tertiary structure. About 20% of the nucleotides are invariant, and in some cases are involved in hydrogen bonds that stabilize the tertiary structure. The common tRNA structure is required because all tRNAs must interact with the ribosome, with elongation factors and with common RNA processing and modification enzymes. However, there must exist some distinguishing features within each set of cognate tRNAs which mediate specific interaction with the corresponding aaRS. In*E.coli*, there are about 45 tRNA species and 20 aaRS in *E.coli* (3), so of the 900 possible interactions between tRNAs and synthetases, only 45 are actually correct, 5% of the total. The recognition problem is further complicated by a need for some flexibility in tRNA-synthetase recognition. Because of degeneracy in the genetic code, more than one isoacceptor tRNA must usually be recognized by a single synthetase. Thus, there must exist unique elements common to isoacceptor tRNAs recognized by the

cognate synthetase and not recognized by the other 19 aaRS. The cell has evolved to perform this task remarkably well. The error rate in protein synthesis is about 10^{-4} per step (4).

What features of the three-dimensional tRNA molecule confer aaRS specificity? Because of the complexities of RNA structure, recognition can take place not only by direct base interactions, but also by contacts with the sugar-phosphate backbone. Additionally, the protein may recognize idiosyncratic features of the overall shape of the tRNA. There are two types of recognition elements: positive elements, which directly interact with the cognate aaRS or position other elements, and negative elements, which discourage interactions with non-cognate aaRS. These positive and negative elements for any isoacceptor group make up its identity set, and the subset of only the positive elements make up its recognition set. As discussed below, positive recognition elements can be determined by *in vitro* and *in vivo* experiments while negative elements can in general only be determined in vivo in the context of the competition of the 20 aaRS for a particular tRNA.

In order to understand the identity problem in protein synthesis, a careful examination of each player in the process is necessary. In the next section I will review our current understanding of the sequence and structure of both the tRNA and the synthetases.

Transfer RNA Structure

The primary sequence information of tRNA is conveniently displayed in a standard RNA cloverleaf (5) (figure 1) illustrating the Watson-Crick base pairing between complementary sequences forming

the secondary structure of the tRNA. Each tRNA contains four helical "arms." The D-arm contains 3 or 4 base pairs in the stem and a loop which varies in length from 7 to 10 bases. The T-arm and the anticodon arm contain a 5 base pair stem and a loop of 7 bases. The nucleotides at positions 34, 35, and 36 are the anticodon. The acceptor arm consists of a stem of 7 base pairs (except $tRNA^{His}$ which has an extra base pair) and an unpaired sequence of four bases whose 2' or 3'-OH group is aminoacylated. Distinctive features of the single-stranded sequence are the terminal CCA, universally invariant among all the tRNAs, and the base at position 73, the discriminator nucleotide (6).

The crystal structure of yeast $tRNA^{Phe}$ (7, 8). (figure 2) reveals that most of these invariant nucleotides are involved in hydrogen bonding interactions which are required to maintain the tertiary structure. The hypothesis is supported by the crystallographic data for yeast $tRNA^{Asp}$ (9, 10), *E.coli* $tRNA^{Gln}$ (11), $tRNA^{Met}$(12),and *T.thermophilus* $tRNA^{Ser}$ (13). In all of these the crystal structures the tRNA forms a similar L shaped structure created by the intersection of two double helices whose axes subtend an internal angle of about 90^{o} (figure 2 (10)). The T-arm and the acceptor arm form one coaxial helix; the D-arm and the anticodon arm form the other one. The double helices intersect in a central domain which contains the T-loop and the D-loop. Tertiary interactions in the single stranded D and T-loops between the invariant bases (G18-Y55, G19-C56, A9-A23, T54-A58, U8-A14), and the semi invariant bases [(positions 15-48 (the Levitt base pair), positions 9-23-12, and positions 13-22-46 (not present in type II tRNA, because of the extra arm)] establish the central core and are

responsible for its 3-dimensional folding shown on figure 2. A group of bases in this domain which differ among the different isoacceptors groups, form a variable pocket ((7, 8). In yeast $tRNA^{Phe}$ these are bases 16, 17, 20, 59 and 60. Because this region is variable and on the surface of the molecule it was suggested that the variable pocket could be important in aaRS recognition(8). Among the different tRNAs there is only slight variation within these sequence and structural constraints, and these variations are responsible for accurate tRNA synthetase recognition. The differences between tRNAs are in the sequence of their anticodons, the sequences of the base-paired arms and in the discriminator base, N73, in the length and sequence of the extra-arm, in the sequence and organization of the variable pocket and in the sequence of the bases in the D-loop. The α region (positions 16,17, and 17a) and the ß region (positions 20, 20a, and 20b) of the D-loop, through tertiary interactions, determine the structure of the variable pocket so these bases can determine specificity by affecting tertiary structure. Thus the recognition of tRNA could be via one, or more likely a combination, of these distinctive features.

Aminoacylation takes place at one end of the L-shaped molecule, and the anticodon loop is at the other end 80 Å away in the tertiary structure. This means that recognition of the anticodon would require that the aaRS be at least this length. As we shall see the aaRS are, in fact, elongated, multi-domain proteins which often do, in recognizing the tRNA, span its entire length.

Aminoacyl tRNA Synthetase Structure

The aaRS constitute a family of enzymes that vary in size and quaternary structure, and yet are responsible for the same function, the recognition and ATP-dependent acylation of tRNA. This occurs in two steps. In the first step the aaRS binds ATP and the amino acid and forms a mixed anhydride between the amino acid and AMP, aminoacyl AMP, with the release of pyrophosphate. This reaction requires magnesium ions. This step can typically be carried out in the absence of tRNA, with the exception of GlnRS, GluRS, and ArgRS where the aaRS need to bind the tRNA before being able to bind ATP (14). In the second step the tRNA is aminoacylated at its 3' terminus, and released from the enzyme.

On the basis of sequence similarities, the aaRS can be divided into two classes (table 1) (15-17). This classification is also correlated with the ability of the aaRS to attach the amino acid to either the 2'-hydroxyl group of ribose (class I) or the 3'-hydroxyl group (class II, with the exception of PheRS). The crystal structures of 11 different aminoacyl-tRNA synthetases with and without their substrates have been solved(18): MetRS (19), TyrRS (20) , GluRS (21), TrpRS (22), and GlnRS (23) in class I, SerRS (13), AspRS (9), LysRS (24), HisRS (25), GlyRS (26), and PheRS (27) in class II. Class I aaRS contain an active site that is a Rossman nucleotide binding fold formed by a ß-sheet of five parallel strands, surrounded by α helices. Class II aaRS contain an active site domain formed by a ß-sheet of six antiparallel strands , in contact with two α helices. Two class II co-crystal structures and one class I have been solved. These are *E.coli* GlnRS-tRNA^{Gln} (11) ,yeast AspRS-tRNA^{Asp} (9) , and *T.theromphileus*SerRS tRNA^{Ser} (13, 28). The

structural differences in the active site correlate with the direction from which the aaRS approaches the tRNA molecule. Class I aaRS approach the tRNA from the D-loop side, establishing contacts with the anticodon and the minor groove of the acceptor stem. Thus the single-stranded CCA 3' must make a sharp turn in order to enter the active site. Class II aaRS approach the tRNA from the variable arm side and also (with the exception of AlaRS and SerRS) interact with the anticodon. However, they contact the major groove of the acceptor stem, thus allowing the CCA 3' to fit into the active site without any distortion of the helical conformation. The co-crystal structures also illustrate that the enzymes are organized in "modules" or domains. In general each domain binds a characteristic recognition site in the tRNA(29).

A Perspective on tRNA-Synthetase Recognition

Elucidating the mechanism of tRNA-synthetase recognition requires a combination of approaches. Our present understanding of the molecular mechanisms responsible for the recognition of tRNAs by their cognate aaRS is essentially based on three sources of information: a) the characterization of tRNA identity determinants b) the structural results of crystallographic investigations and c) physical chemical studies of the interactions between tRNA and aaRS in solution. .

The Characterization of tRNA Identity Determinants

Two main experimental approaches have been used to determine the recognition elements that distinguish one set of tRNAs from all others. Each method has its advantages and constraints. Although

different kinds of information are derived from the approaches, the results from the two approaches often agree though, of course, negative identity elements cannot be explained by the results from studies of cognate pair interactions.

The *in vivo* Approach

This method is based upon previous work that has been reviewed in detail elsewhere (30). Transfer RNA suppressors for the amber codon were used three decades ago to study the altered specificity of genetically isolated *E.coli* $tRNA^{Tyr}$ amber suppressor mutants. Altered specificity with single base mutants was observed and it was exclusively a change to glutamine identity(31, 32) . Because it was technically unfeasible to genetically isolate multiple mutations at will, additional identity changes could not be identified at the time.

However advances in oligonucleotide synthesis made it possible to construct genes of variant tRNA suppressors by annealing and ligating different oligonucleotides and cloning them into a plasmid under the control of an *E.coli* promoter to be constitutively transcribed (33, 34). The biological functionality of the mutant is established by analyzing the efficiency with which the tRNA was able to suppress an amber mutation in the lacZ gene when the tRNA gene is under the control of the *E. coli* lpp promoter. This is called an efficiency assay (35). The amino acid identity of the tRNA suppressor is determined by sequencing the protein product of a reporter gene containing an amber mutation. Our laboratory has used an amber mutation at position 10 in the *E.coli*

dihydrofolate reductase gene for this purpose. The amino acid residue of the suppressed DHFR at position 10, sometimes a single amino acid, sometimes a mixture is determined by the identity of the amber suppressor gene that is being examined. This assay is an "identity assay" (36). Both amber mutations in the two reporter genes are engineered at positions that are away from the catalytic site of the protein, and therefore any amino acid can be accepted without altering the functionality of the mutated protein. These studies have allowed the identification of the minimal number of base changes needed to cause one tRNA species to be selectively aminoacylated by a non-cognate synthetase. This is called an "identity swap" experiment. The set of these minimal changes has been defined as the identity set. The identity set includes the positive elements for one aaRS and the negative elements for the remaining 19.

Unfortunately, it is difficult to directly correlate the results of the efficiency and the identity assays with *in vitro* aminoacylation results using pure tRNA and aaRS. The efficiency assay is not only the result of how well a tRNA suppressor is aminoacylated, but also how well it is processed and modified, and how well it interacts with the translational apparatus. The specificity assay reflects the outcome of the competition for aminoacylation of the tRNA by all 20 aaRS.

The *in vitro* Approach

The development of an *in vitro* transcription method by which transcripts from synthetic tRNA genes could be obtained has allowed the creation of any desired tRNA variant and its recovery (as an unmodified

transcript) in large quantities (37). The fact that these transcripts are purified in denaturing conditions and that they do not have secondary modifications, does not seem to prevent their correct refolding into the active conformation. These substrates allow quantitative comparisons to be made of the effects of base changes at specific recognition sites by purified aaRS, within the range of feasible enzyme and substrate concentrations.

Applying both the *in vitro* approach, determining the recognition set, and the *in vivo* approach to determining the identity set elucidates which specific interactions between a tRNA and aaRS are crucial. A lot of progress in elucidating the tRNA identity rules has been made in recent years (30, 38-43). We now have information about how all 20 sets of E. coli tRNA are recognized. The recognition elements for the aaRS have been located in the anticodon, the acceptor stem and the discriminator base at position 73, and the central core. Often the identity of a particular tRNA is determined by elements in more than one of these regions.

Recognition of the Anticodon

The anticodon is a single-stranded region with bases available for interaction. Transfer RNAs from different isoacceptor groups have different anticodon sequences as dictated by the genetic code and the "wobble rules" (44). Therefore it is logical that the anticodon is one of the recognized features (45-51).

The Abelson laboratory in collaboration with the laboratory of Jeffrey Miller at UCLA undertook a project to synthetically create a

complete set of amber suppressors (33, 34) As it turned out all amber suppressors could not be made because the CUA anticodon required to recognize the UAG amber codon was not compatible with recognition by some aaRS. Substitution of the original anticodon with the amber suppressor anticodon in 20 isoacceptor groups of tRNAs results in an identity change for 9 isoacceptor groups (see table 2). Most of them are misacylated with glutamine or lysine, indicating that a U at position 35 is a positive element for GlnRS and LysRS and subsequent research confirmed this for GlnRS (52, 53) and for LysRS (54). In the case of the isoacceptor groups that retain the original identity, there are 3 possible explanations: a) the cognate aaRS does not recognize the anticodon; b) there are sequence homologies between the original anticodon and CUA; or c) there are strong negative elements for GlnRS and LysRS. Transfer RNA^{Ala}, $tRNA^{Ser}$, and $tRNA^{Leu}$ have been characterized *in vitro* and have been shown to lack detectable anticodon-dependent aminoacylation (55-57). In these cases, the wild-type anticodons might actually function as negative elements for the other aaRS. In the case of the amber suppressor $tRNA^{Cys}$ (58), $tRNA^{Phe}$ (59), and $tRNA^{Gln}$ (53) it has been shown that even if the *in vivo* identity is retained, the aminoacylation of the tRNA by the cognate aaRS is decreased with respect to the wild-type tRNA.

Mutation of the anticodon results in significant reduction of *in vitro* aminoacylation for 17 of the isoacceptor groups (table 3). As expected from the *in vivo* data, the effects vary between the different groups. Single nucleotide substitution has a major effect in some cases (table 3), in other cases, however, more than one nucleotide must be

changed in order to see an effect (table 3), implying that the sum of the combined contribution of the three nucelotides of the anticodon is important for recognition.

The fact that the transcribed tRNAs do not contain the secondary modifications otherwise present in the cell illustrates the necessity of certain modified bases for the correct interaction with the aaRS. Secondary modifications in the anticodon of $tRNA^{Ile}$ (lysidine at position 34) (60, 61) and $tRNA^{Glu}$ (2-thiouridine at position 34) (62) are positive elements directly contacted by the cognate aaRS. A single methyl group modification of G37 is a negative element preventing mischarging of $tRNA^{Asp}$ (63) by yeast ArgRS, *in vitro* T7 transcripts of wild-type $tRNA^{Arg}$ and $tRNA^{Asp}$ are aminoacylated with equal efficiency.

The Acceptor Arm

The aaRS generally have at least two major domains which interact with the tRNA. The catalytic domain which comprises the interclass conserved active site, interacts with the acceptor arm of the tRNA. The other domain contacts the anticodon and its coaxial helix. Even though the majority of aaRS interact with the anticodon, introducing mutations in the acceptor stem and the discriminator base of these tRNAs often affects aminoacylation both *in vivo* and *in vitro* (49). Several tRNAs containing mutations in the anticodon are still charged with the original amino acid or with a mixture of amino acids, suggesting that there are additional recognition elements outside of the anticodon of these tRNAs.

"Minihelix" constructs consisting of the acceptor stem and the T-stem and loop and "microhelices" (comprised of only the acceptor arm) derived from the sequences of the cognate tRNAs can be aminoacylated in an anticodon independent manner by the HisRS, AlaRs, GlyRS (64) , SerRS (65), MetRS (66), GlnRS (38), ValRS (67), AspRS (68), and IleRS (68). The role of the acceptor stem in aminoacylation specificity is sequence-specific and depends on the accessibility of the base pairs of the A-helix. The acceptor stem forms an A-RNA helix with a deep and narrow major groove, inacessible, and a wide and shallow minor groove, easily accessible (69). According to the model, deduced from the three co-crystal structures, the class I aaRS should interact with the minor groove, and the class II aaRS with the major groove.

The class I aaRS, GlnRS contacts $tRNA^{Gln}$ in the minor groove, and in this specific case the interaction is also made possible by the melting of the base pair at the end of the helix (see the discussion of the GlnRS-$tRNA^{Gln}$ co-crystal below) (11).

Class II aaRS contact their cognate tRNAs in the major groove, where base specific interactions are not available except for the base pairs close to the end of the acceptor stem helix (70), and they solve this accessibility problem in different ways.

AspRS makes specific base contacts only with the single stranded discriminator and base pair A1-U72 and by forming hydrogen bonds with the phosphate backbone of the acceptor stem base pairs (9).

SerRS interacts with the first two base pairs at the 3'-end of the acceptor stem helix, and makes specific contacts with U68 and C69 by

forming hydrophobic interactions with the ring of residue Phe262, which is perpendicular with respect to the base pairs(28, 65).

AlaRS interacts with the G3-U70 wobble pair G2-C71, and also A73 in the acceptor stem of its cognate tRNA (71, 72). Introducing G3-U70 into $tRNA^{Phe}$, or into RNA mini and micro helices causes these RNAs to be aminoacylated by AlaRS (57, 73, 74)., and substitution of G3 with the base analog inosine (identical to guanosine except for a keto group replacing the 2 -amino group of G) affects aminoacylation by AlaRS (75). The G3-U70 base pair presents an exocyclic 2-amino group in the minor groove that is not involved in hydrogen bonding with the complementary base. Thus this specific interaction takes place on the opposite side of the helix than what is expected for class II synthetases. Crosslinking studies have shown that an insertion of 76 amino acids residues in the catalytic domain makes contact with the exocyclic 2-amino group in G3-U70. This contact is postulated to occur via an extension of the protein in this region which reaches around the helix to contact the minor groove (76).

Central Core

The tertiary interactions between the single stranded D and T-loop invariant nucleotides, forming the central core; the semi invariant nucleotides, forming the Levitt base pair; the non conserved nucleotides in the D-loop, forming the variable pocket; and the D-stem, forming the central part of the molecule are all important for the L-shape structure and may be involved in direct interactions with the aaRS.

Unfortunately, the role of these elements is not always so evident, because of the conformational changes that can be introduced by mutations in these positions. Another misleading factor as far as recognition goes can be a masking effect due to other recognition sites located elsewhere in the tRNA. There are already several examples of aaRS involved in direct recognition of some of these features.

The AspRS specifically contacts G10-U25 in the stem of the D-arm of $tRNA^{Asp}$ (9), but the correct structure of the $tRNA^{Asp}$ is required for the presentation of this feature and efficient aminoacylation. Mutations in positions involved in tertiary interactions in the D-loop, not directly contacted by the AspRS as shown in the crystal structure, alter the presentation of this base pair resulting in a decreased efficiency of aminoacylation and a variation in the chemical protection pattern(77).

Yeast PheRS recognizes G20 in the D-loop and is more sensitive to the correct folding of the molecule than to the sequence that is involved in forming the tertiary interactions. The central core of the tRNA has been studied in detail by systematically mutating nucleotides, invariant and semi invariant, involved in tertiary interactions, as shown on the crystal structure. Substitution of tertiary interactions with the corresponding interactions typical of other tRNA results in substrates that are efficiently aminoacylated. Furthermore, the structural specific lead cleavage pattern is the same as the wild type. It is only when the tertiary structure is changed, destroying the tertiary interactions, that the aminoacylation is affected .

While it was known that *E.coli*$tRNA^{Phe}$ G20 was an important recognition site, it was also thought that nucleotides G10-C25-A-26, and

G44-U45 forming tertiary interactions in the central core were important recognition sites for the *E.coli* PheRS. They appeared to be positive elements of the identity set of $tRNA^{Phe}$ *in vivo* (71). Mutations at these positions caused a decrease in aminoacylation in *in vitro* studies, although the structure specific lead cleavage proved the mutated tRNA to have a correct structure(59), lending support for the notion that sequence specific interactions were crucial. Surprisingly, a variety of sequences were tolerable at these positions. *In vitro* selection of tRNA molecules from a $tRNA^{Phe}$ library randomized at these positions demonstrated the possibility of alternative tertiary interactions. The possibility of a direct contact is therefore excluded, and the role played by these interactions and the newly selected sequences is to direct the correct structure of the tRNA (78).

Of course in some cases, tertiary interactions in the central core play an important role in the recognition. For example, for $tRNA^{Cys}$. Substitution of the Levitt base pair sequence G15-G48, unique to $tRNA^{Cys}$, results in tRNAs with unaltered structure, but affects aminoacylation (58, 79, 80).

Crystallographic Investigations and Solution Studies

Three tRNA-synthetase co-crystal structures have been solved by X-ray diffraction. These structures provide an excellent correlation with the available data from the *in vivo* and *in vitro* studies.

The $tRNA^{Gln}$ / GlnRS complex

The refined crystal structure of *E. coli* glutaminyl tRNA synthetase complexed with $tRNA^{Gln}$ (figure 3) was the first aaRS-tRNA structure solved (11). The diffraction studies show that the catalytic site of class I synthetases form a Rossman-like ATP-binding motif , and shed light on how class I aaRS approach the tRNA (81, 82). There are many interactions taking place between the tRNA and the aaRS, some are base specific; others are interactions with the phosphate backbone that require the presence of a specific base for structural reasons. The structure of the anticodon loop of the tRNA, interacting with GlnRS goes through an evident structural rearrangement, especially if compared with the crystal structure of $tRNA^{Phe}$(7, 8). The anticodon loop appears to be extended by the presence of two stacked, non-Watson-Crick base pairs in the anticodon stem. This results in the protrusion of the three unpaired anticodon nucleotides, essential sites for GlnRS recognition. These bases can form several hydrogen bonds to complementary pockets in the protein. U35, and G36 are directly in contact with the GlnRS as has been demonstrated by extensive *in vitro* studies (23, 83).

The acceptor stem also goes through conformational changes on GlnRS binding. The base-pair U1-A72 is disrupted to allow the single stranded 3'-end to point towards the catalytic site and fit into a deep pocket in the GlnRS. Substitution of the more energetically stable base pair G-C at this position resulted in a decrease in aminoacylation by the GlnRS *in vitro* (84). The role played by this base pair could not have been deduced without the structure. This conformational change is also stabilized by the stacking of A72 and G73. The discriminator base, G73,

positions the 3' end by forming a hydrogen bond between its 2-amino group and the phosphate linking C71 and A72. This RNA-RNA interaction explains the requirement for a G at 73. Thus G73 is an identity element by virtue of its affect on RNA structure and not via its specific interaction with the protein, an important precedent to keep in mind when interpreting the function of identity elements. GlnRS, a class I synthetase, interacts with the minor groove of the acceptor stem contacting the exocyclic 2-amino group of bases G2 and G3,. This interaction has been confirmed by *in vivo* (85) and *in vitro* (53) studies, and is consistent with the sequences of glutamine amber suppressors selected *in vivo* from a tRNA library randomized at the acceptor stem (85). The fact that GlnRS is capable of aminoacylating minihelix substrates is also a further testimony to the important role played in specificity by the acceptor arm (38)

Since the solution of the $tRNA^{Phe}$ structure (7, 8) it has been apparent that the anticodon is a long ways away from the aminoacylation site. Whether, and if so how, the anticodon and the acceptor stem "comunicate" between each other once they interact with the aaRS is still an open question. In an attempt to test the possibility of a functional communication between the two sites, the effect on GlnRS aminoacylation of acceptor arm minihelices by anticodon minihelices was tested. There was no effect unless the minihelices were in cis, although it was a very promising fact that the anticodon minihelices were shown to inhibit the aminoacylation of full length tRNA (38).

$tRNA^{Asp}$ / AspRS complex

The refinement of the crystal structure of the complex formed by yeast AspRS and $tRNA^{Asp}$ (9, 86) (figure 4) has provided an understanding of the recognition of tRNA by class II aaRS. AspRS exhibits the class II characteristic catalytic site domain that have been described in the aaRS section of this review. It contains one catalytic site with a tRNA molecule bound in each of the two identical subunits. In the comparison with the previously solved structure of GlnRS it is obvious that AspRS approaches the tRNA molecule from the opposite side-from the variable loop side and interacts with the major groove of the acceptor stem. The X-ray diffraction studies show that three "modules" of AspRS are in contact with the recognition sites of $tRNA^{Asp}$. As in the case of $tRNA^{Gln}$, $tRNA^{Asp}$ goes through conformational changes upon binding its cognate aaRS. The novelty in the case of $tRNA^{Asp}$ is that the structure of $tRNA^{Asp}$ alone has been solved (10). This allows comparison between the tRNA structure before and after binding to AspRS. The anticodon loop, as in $tRNA^{Gln}$, undergoes a conformational change. The three nucleotides G34, U35, and C36 in the anticodon directly interact with the protein. The base G10 is also contacted in the D-arm of the tRNA. Contrary to what is seen in $tRNA^{Gln}$, the acceptor stem does not go through major conformational changes and only the discriminator base G73 and base U1-A72 are specifically contacted by AspRS. The other contacts with the protein are with the phosphate backbone of the single-stranded 3'-end four nucleotides of the acceptor arm. Because of the way it approaches the tRNA, the AspRS contacts the major groove of the acceptor stem

explaining why a conformational change in the 3'-CCA is not necessary for the terminal A to fit into the catalytic site.

The importance of these recognition sites has been confirmed by *in vitro* aminoacylation studies of T7-transcribed $tRNA^{Asp}$ mutants and also by chemical protection studies of iodine cleavage of phosphorothioate-substituted transcripts in the presence or the absence of AspRS. Introducing mutations at the recognition sites G73, G10-U25, G34, U35, and C36 (with the exception being base pair U1-A72 where changing it to G-C does not alter the aminoacylation) leads to a decrease in aminoacylation and also results in the protection pattern varying only in the position mutated. Interestingly, introducing these recognition sites for AspRS into $tRNA^{Phe}$, not only transforms it into a substrate for AspRS aminoacylation but when the tRNA is tested for chemical protection gives the same protection pattern of wild-type $tRNA^{Asp}$(87) (88) . RNA mini and micro helices of the acceptor stem of yeast $tRNA^{Asp}$ are aminoacylated by the AspRS. Introducing changes in any of the acceptor stem positions does not affect the aminoacylation of the helix; only changing position G73 results in a loss of aminoacylation activity. The AspRS is insensitive to the acceptor stem sequence, only contacting the phosphate backbone. The fact that a minimal substrate consisting of only a tetra loop, three bases pairs, and the G73 is by the AspRS confirm this (68).

$tRNA^{Ser}$ / SerRS complex

The most recently solved crystal structure is*T. thermophilus* seryl-tRNA synthetase with $tRNA^{Ser}$(13) (figure 5). The synthetase is a

dimer but only one tRNA molecule is bound in the complex. Most class II aaRS are dimers and thus have two catalytic sites, one for each subunit, but the way in which the dimer binds tRNA in SerRS is different from that in AspRS. $tRNA^{Ser}$ interacts with both subunits; $tRNA^{Asp}$ with only one. SerRS, a class II synthetase like AspRS, recognizes the major groove of the acceptor stem, making base specific contacts with the base pairs G1-C72, G2-C71, G4-C69, and A5-U68 on one subunit, and with G47a-C47m in the extra arm and G19 in the D-loop of $tRNA^{Ser}$ on the other subunit. The interaction of SerRS with the extra arm of the tRNA is peculiar to this system, and takes place via a striking protein structural domain formed by an antiparallel coiled-coil. Position G19 not in direct contact with the SerRS in the co-crystal is fundamental importance for the correct positioning of the extra-arm. Another peculiarity about this system is that SerSR is not involved in interactions with the anticodon of the tRNA. The refined structure of the SerRS alone(89), enables us to compare the confomational changes that are introduced into the protein upon tRNA binding (28).. The GlnRS coil-coil domain goes through a conformational change upon binding to the extra arm of the tRNA. The binding of the tRNA extra arm is a necessary first recognition step for efficient aminoacylation to take place in the catalytic site, where residues bound to the aminoacyl-AMP by changing orientation alter the order of the structure.

The different arms of *E.coli* $tRNA^{Ser}$ have been tested for their role in recognition by examination of the effects of systematic deletions on in vitro aminoacylation by SerRS (55). The removal of the anticodon arm resulted in a tRNA with kinetics parameters identical to that of wild

type, supporting the notion that the anticodon is not involved in any interaction. The removal of the extra arm and its replacement with a typical type I tRNA loop consensus resulted to serine identity loss in *in vivo*(90), and a substantial decrease in aminoacylation by the SerRS *in vitro* (55) . Phosphorothioate footprinting experiments also indicate direct contacts with nucleotides in the extra-arm and also the acceptor stem (91). Experiments in this thesis also show how crucial the orientation of the extra arm is for correct recognition by SerRS.

An acceptor arm sequence in a minihelix substrate is aminoacylated by SerRS, allowing a study of the contribution of acceptor stem base pairs to recognition otherwise not immediately evident in full length $tRNA^{Ser}$. The general picture of bases recognized in the acceptor stem was more detailed than that obtained from the initial crystal structure because this region was not resolved due to local disorder, in fact the interaction between $tRNA^{Ser}$ and SerRS is not only influenced by contacts with bases 4-69 and 5-68, but is also influenced by the base pair sequence at position 1-72, 2-71, and 3-70 (65). The high resolution structure studies confirmed these results and elucidated a novel interaction taking place between the ring of Phe262 residue and the rings of the bases at position bases U68 and U69, and the function of A3-U70 necessary for the correct interaction with the recognition site base pairs(28).

Figure 1. tRNA Secondary Structure.

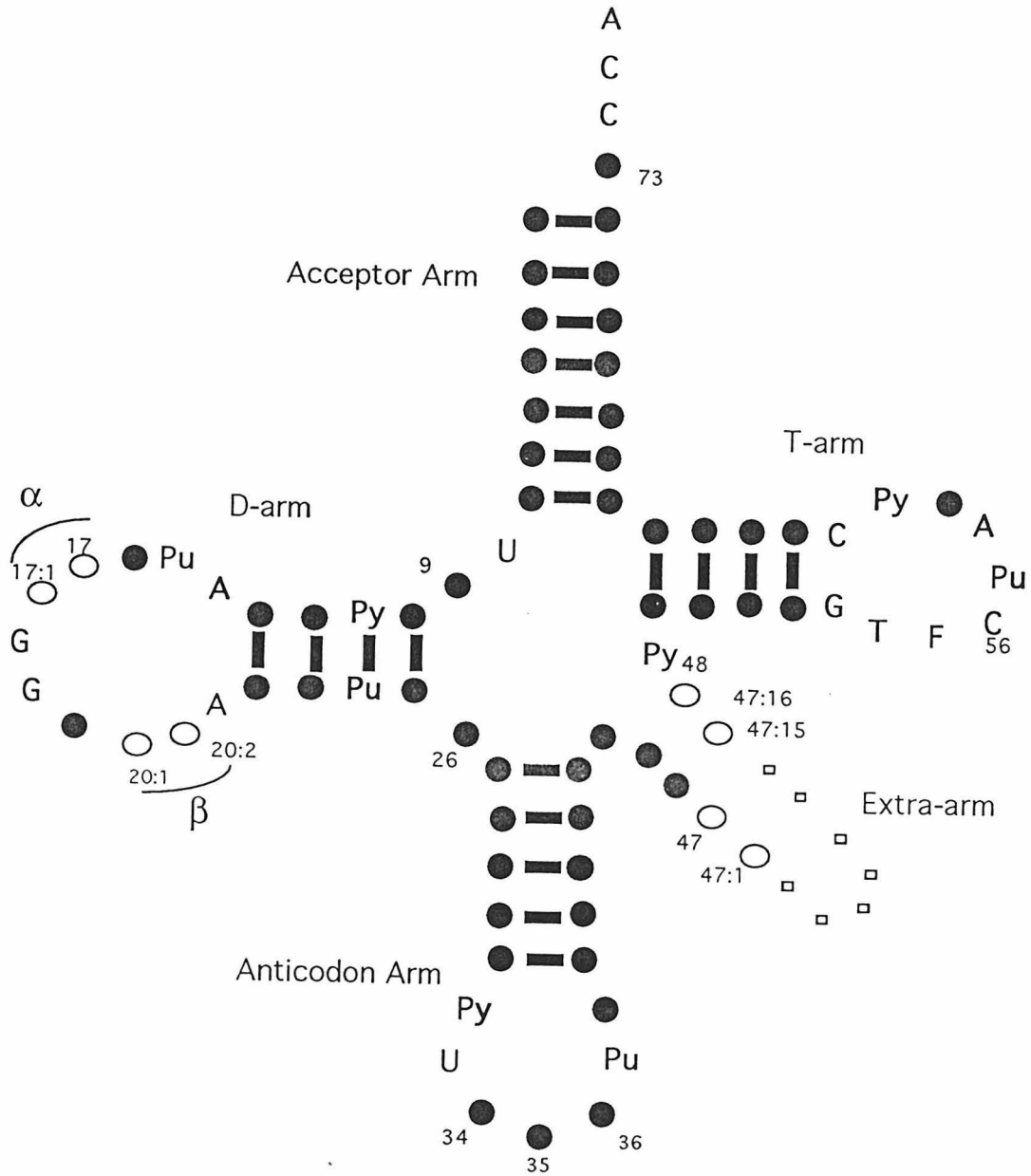
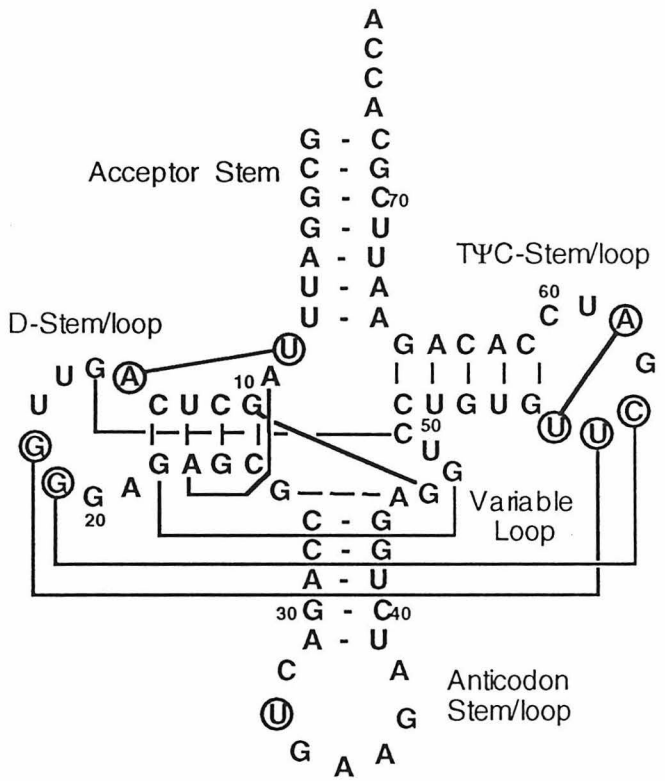

A
C
C
73
Acceptor Arm
T-arm
α
D-arm
17
17:1
Pu
9
U
Py
A
C
Pu
G
A
Py
Pu
G
T
F
C
56
Py 48
47:16
47:15
A
20:2
20:1
β
26
Extra-arm
47
47:1
Anticodon Arm
Py
U
Pu
34
35
36

Figure 2. Yeast $tRNA^{Phe}$ secondary structure. The invariant nucleotides involved in tertiary structure are indicated. Tertiary structure of type I tRNA (bottom left). Tertiary structure of type II tRNA (bottom right).



Type I

Type II

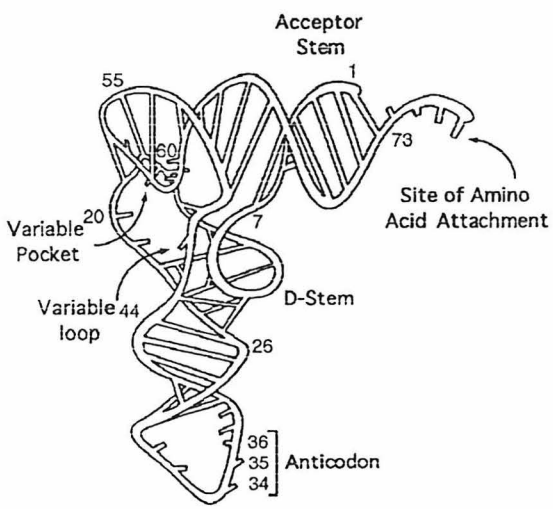


Figure 3. *E.coli* $tRNA^{Gln}$ complexed with GlnRS (11).

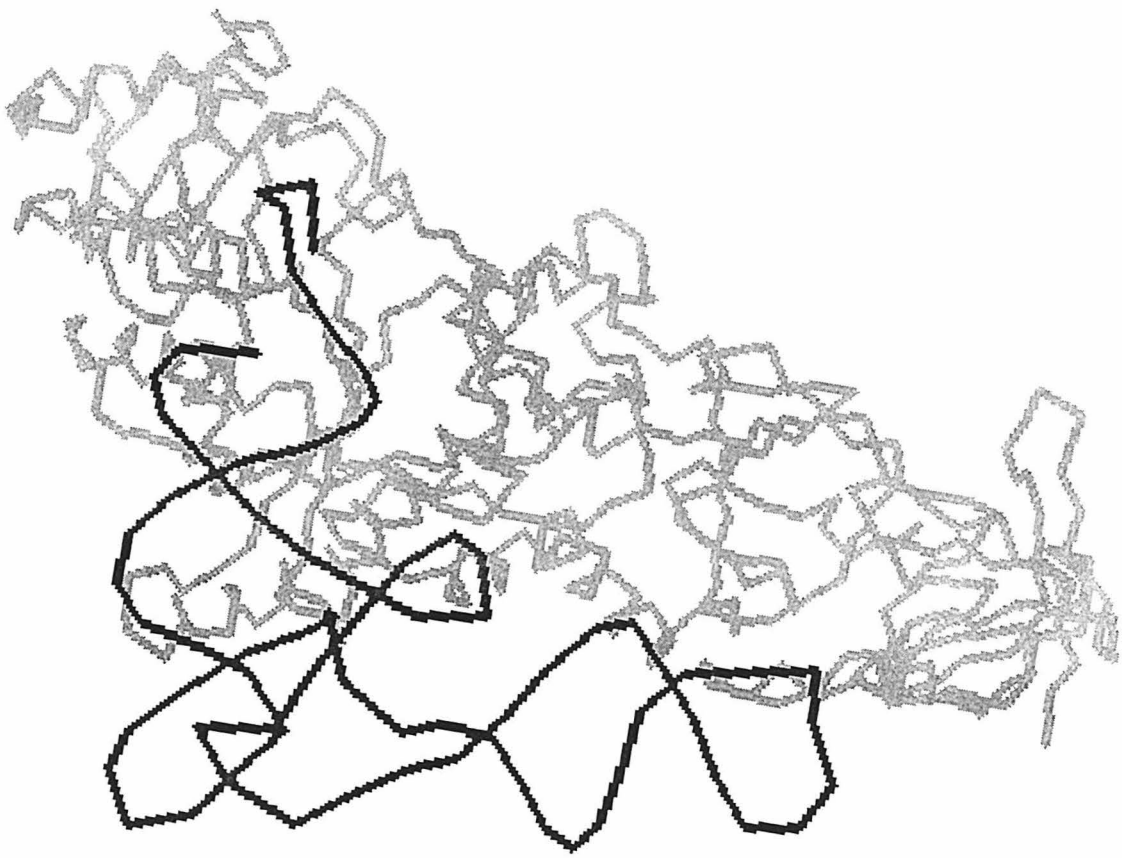


Figure 4. Yeast $tRNA^{Asp}$ complexed with AspRS (9).

Figure 5. *T.thermophilus* $tRNA^{Ser}$ complexed with SerRS (13).

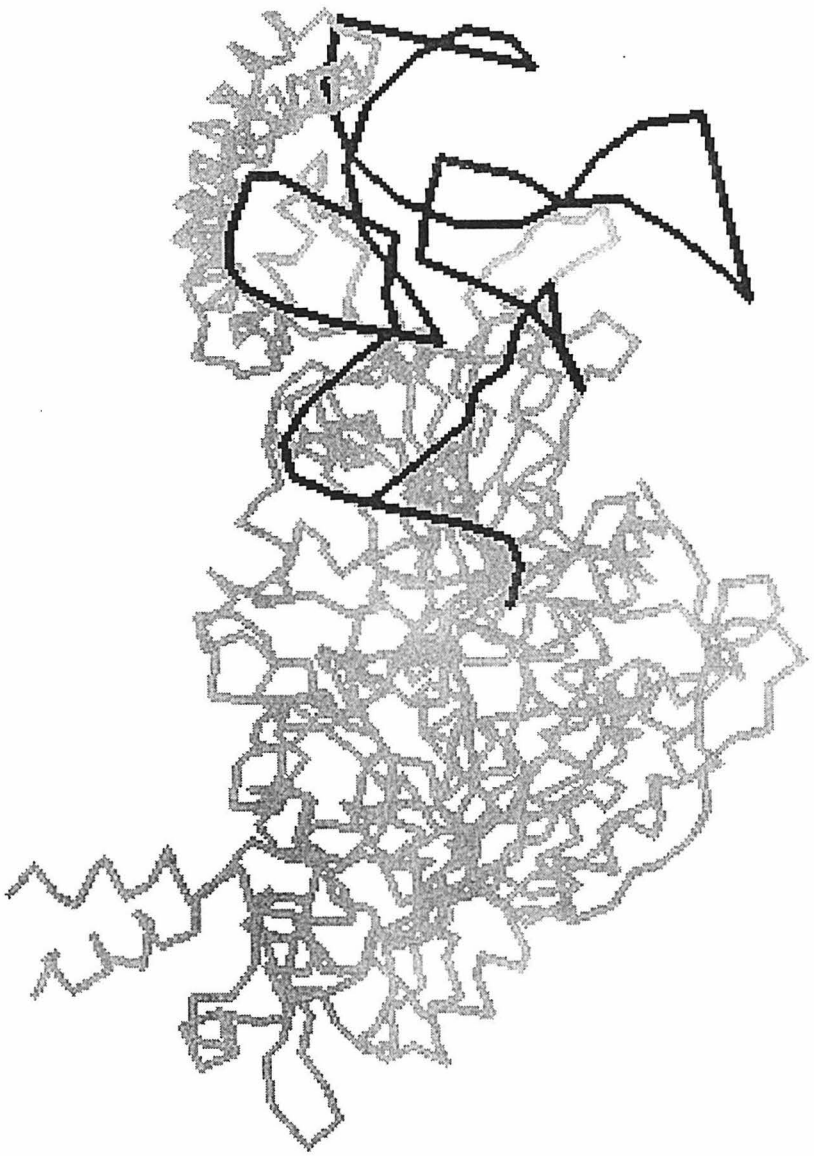


Table 1. Characteristics of aminoacyl-tRNA synthetases.

Table 1. Aminoacyl-tRNA Synthetases

aaRS	Class	Oligomeric Structure	Site of a.a. attachment	No. catalytic sites	Refs.
Ala	II	α_4	3'-OH		(92)
Arg	I	α	2'-OH		(93)
Asn	II	α_2	3'-OH		(94)
Asp	II	α_2	3'-OH	2	(9)
Cys	I	α	2'-OH		(95)
Gln	I	α	2'-OH	1	(11)
Glu	I	α	2'-OH		(96, 97)
Gly	II	$\alpha_2\beta_2$	3'-OH		(26)
His	II	α_2	3'-OH	2	(25, 26, 98, 99)
Ile	I	α	2'-OH	1	(100)

Table 1 cont.

aaRS	Class	Oligomeric Structure	Site of a.a. attachment	No. catalytic sites	Refs.
Leu	I	α	2'-OH		(101)
Lys	II	α_2	3'-OH		(24)
Met	I	α_2	2'-OH	2	(19)
Phe	II	$\alpha_2\beta_2$	2'-OH	2	(27)
Pro	II	α_2	3'-OH		(102)
Ser	II	α_2	3'-OH	2	(13, 28)
Thr	II	α_2	3'-OH		(103)
Trp	I	α_2	2'-OH	1	(104)
Tyr	I	α_2	2'-OH	1	(20)
Val	I	α	2'-OH	1	(105)

Table 2. Summary of amber suppressor identities obtained from (30).

Table 2. Amber Suppressor tRNA Genes
(Classified by effect of a CUA upon aaRS Recognition)

Class I No affect upon charging	Class II GlnRS mischarging	Class III LysRS mischarging
Ala2	GluA	Arg
GlyU	Gly2	AspM
Cys	Ile1	Ile2
Phe	fMet	Met
ProH	Trp (su^+7)	Thr2
HisA		
Lys		
Ser (su^+1)		
Gln (su^+1)		
Tyr (su^+3)		
Leu (su^+6)		

Table 3. Known recognition elements.

Table 3. Summary of Known Recognition Elements

tRNA	Anticodon	Discrim. Base	Acceptor Stem	D-stem Loop	X-Arm Recog.	Refs.
Ala	None	A_{73}	G_2-C_{71}, G_3-U_{70}			(106)
Arg	C_{35}	A_{73}		A_{20}		(107, 109)
Asn	$G_{34}U_{35}U_{36}$	G_{73}				(110, 111)
Asp	$G_{34}U_{35}C_{36}$	G_{73}		G_{10}-U_{25}		(9, 77, 87, 88, 112, 118)
Cys	$G_{34}C_{35}A_{36}$	U_{73}		G_{14}-G_{48}		(119, 122)
Gln	$R_{34}U_{35}G_{36}$	G_{73}	G_2-C_{71}, G_3-U_{70}			(52, 53, 83, 123, 124)
Glu	U_{34}	G_{73}				(49, 62)
Gly	$C_{35}C_{36}$	U_{73}	G_2-C_{71}, G_3-U_{70}			(125, 126)
His	U_{35}	C_{73}	G_1-C_{73}, U_2-A_{71}, G_3-U_{70}			(64, 127)
Ile	$L_{34}A_{35}U_{36}$	A_{73}	C_4-G_{69}			(49, 128, 129)

Table 3 cont.

tRNA	Anticodon	Discrim. Base	Acceptor Stem	D-stem Loop	X-Arm Recog.	Refs.
Leu	None	A_{73}	G_2-C_{71}, G_3-U_{70}	A_{15}-U_{48}	No	(56, 130, 131)
Lys	$U_{34}U_{35}U_{36}$	A_{73}				(109)
Met	$C_{34}A_{35}U_{36}$	A_{73}	G_2-C_{71}, G_3-U_{70}, U_4-A_{69}			(42, 50)
Phe	$G_{34}A_{35}A_{36}$	None		G_{20},U_{59}, G_{10}-C_{25}, A_{26}, G_{44}, U_{45}		(37, 59, 71, 78, 132, 133)
Pro	$G_{35}G_{36}$	A_{73}	C_1-G_{72}	G_{15},G_{48}		(134, 136)
Ser	None	None	G_2-C_{71}, G_4-C_{69}, A_5-U_{68}		Yes	(55, 65, 90, 130, 137, 138)
Thr	$G_{35}U_{36}$	None				(49)
Trp	$C_{34}C_{35}A_{36}$	G_{73}				(49, 139)
Tyr	$Q_{34}U_{35}A_{36}$	A_{73}			Yes	(138)
Val	$A_{35}C_{36}$	A_{73}				(50, 129, 140)

E.coli tRNA Leucine Identity and Recognition Sets

Introduction

The high degree of accuracy of the translation process is primarily the result of specific interactions between aminoacyl-tRNA synthetases (aaRS) and tRNA. Each of the 20 different aaRS recognizes its own set of cognate tRNAs and does not misacylate non-cognate tRNAs. All tRNAs are similar at the level of primary, secondary, and tertiary structure. About 20% of the nucleotides are invariant, and in some cases are involved in hydrogen bonds that stabilize the tertiary structure. The common tRNA structure is required because all tRNAs must interact with the ribosome, with elongation factors and with common RNA processing and modification enzymes. However, there must exist some distinguishing features within each set of cognate tRNAs which mediate specific interaction with the corresponding aaRS.

The results of studies aimed at defining the recognition properties of the various isoacceptor groups in *E. coli* have been summarized (30, 39-43). The anticodon plays a fundamental role in recognition in 17 out of the 20 isoacceptors groups. Recognition elements may be present in the variable pocket, the acceptor stem, and in the extra arm of two of the three class II tRNAs. Generally, isoacceptor groups are recognized in at least two of these locations, in most cases the acceptor stem and the anticodon.

The nucleotides which determine specificity in the interaction between tRNAs and their cognate aaRS have been identified by two

different methods. An *in vivo* method utilizes amber suppressor tRNAs and measures the efficiency and specificity of sequence variants of the amber suppressor. Efficiency, an indirect measurement of the activity of the tRNA in both the aminoacylation reaction and in protein synthesis, is measured by determining the extent to which a particular amber mutation, typically in the *lacZ* gene, is suppressed relative to the wild-type suppressor (35). Specificity is measured by determining which amino acid(s) is inserted in the suppression of a particular amber mutation in a reporter gene. This is done by determining the amino acid sequence of the suppressed protein. In our laboratory we have routinely used an amber mutation in position 10 of *E. coli* dihydrofolate reductase for this purpose (36). Frequently, identity elements have been determined via an identity swap experiment. In this experiment an attempt is made to completely change the identity of one tRNA, e.g. $tRNA^{Leu}$ in our first experiments, to another identity, $tRNA^{Ser}$ in that case (36). When that can be done, it *per force*, results in the identification of important identity elements though not necessarily the complete set. The *in vitro* method for determining specificity involves the quantitative measurement of the kinetic parameters for the aminoacylation of variant tRNAs by a particular aaRS, often purified. This was facilitated by the development of procedures for the *in vitro* transcription of tRNAs using T7 RNA polymerase (37, 108). Both methods have been extensively employed and together they have given us the rather comprehensive picture of aaRS specificity that has emerged in the past ten years. In general, the two methods agree but not always and

this is because they measure different though overlapping features of protein synthesis.

The *in vitro* method measures the kinetics of the aminoacylation reaction but under conditions of salt, substrate, and enzyme concentration which may be quite different from that found in the cell (22). Furthermore, the rate-limiting step *in vitro* may not be relevant *in vivo* where the tRNA and aaRS concentrations may be very high. The *in vivo* method measures the outcome of the complex and important competition between all twenty aaRS for the same tRNA (4, 24, 44, 141-143). However, the method cannot tell whether poor suppression is due to a failure to interact productively with the cognate aaRS or instead reflects a block in one of the many other steps leading to the eventual suppression step. These include transcription, processing and stability of the tRNA, interaction with elongation factor EF-Tu and the competition with the release factor in protein synthesis. A further complication of the *in vivo* method is that the anticodon of the tRNA must be CUA, recognizing the amber codon UAG. Since for most tRNAs, the anticodon is a crucial recognition element, this method in some cases would examine the identity of a tRNA which is impaired to begin with.

To distinguish between the information obtained by the *in vivo* and *in vitro* approaches, workers in this field have adopted the term "identity elements" to distinguish those bases or structural features of a tRNA which establish its specificity *in vivo* . These identity elements, can be either "positive" or "negative". Positive elements promote recognition by the cognate synthetase whereas negative elements are bases or

structural features which prevent the recognition of a tRNA by a non-cognate aaRS. The "identity set" is the collection of identity elements in a set of tRNAs which allow those tRNAs to be recognized correctly by the cognate aaRS. The term "recognition elements" refers to those bases or structural features which mediate specific recognition of a tRNA by its cognate aaRS in the *in vitro* aminoacylation reaction and the "recognition set" the collection recognition elements which confer in vitro specificity to a tRNA. Note that "negative identity elements" can in general only be identified through the *in vivo* approach.

In cases where the two methods can be optimally employed, valuable complementary information can be obtained by determining both the "identity set" and the "recognition set" for a particular tRNA. This is the approach that we have taken in the study of serine identity, where extensive *in vivo* and *in vitro* studies have led us to an understanding of both the identity elements and recognition elements for this set of tRNAs. In this paper we employ both methods in a study of *E. coli* $tRNA^{Leu}$ aaRS recognition. $tRNA^{Ser}$ and tRNALeu are two of only three sets of tRNAs in *E. coli* for which the anticodon is not a recognition element (the other being $tRNA^{Ala}$; (57)). Thus, they are uniquely suited for the valuable dual study that we have taken. Alleles of both $tRNA^{Leu}$ and $tRNA^{Ser}$ were originally found as amber suppressors. For both $tRNA^{Ser}$ and $tRNA^{Leu}$ there are six codons and therefore, in each case, sets of tRNAs with different anticodons are required. Both tRNAs are designated type II because they both possess a long base-paired extra stem loop, a feature of only the serine, leucine,

and tyrosine tRNAs in *E. coli*. As such, these tRNAs are ideally suited for identity swap experiments. In addition, to these reasons we were particularly interested to study $tRNA^{Leu}$ identity because the information we have already obtained for $tRNA^{Ser}$ (36, 55, 65, 90) could be complemented with information of the $tRNA^{Leu}$ identity set to provide an understanding of how these similar type II tRNAs are distinguished by their respective synthetases in the milieu of the cell.

Considerable information on the recognition set of $tRNA^{Leu}$ has already been published by Asahara et al. (130). These workers studied the *in vitro* aminoacylation of a number of $tRNA^{Leu}$ variants and also carried out two *in vitro* experiments in which $tRNA^{Ser}$ and $tRNA^{Tyr}$ sequences were changed to yield variants with quite significant $tRNA^{Leu}$ specificity. From their results, the following conclusions emerged: 1) The anticodon is likely not to be a recognition element since changing the anticodon of $tRNA^{Leu}_{CAG}$ to $tRNA^{Leu}_{UGA}$ (a serine anticodon) did not significantly affect recognition by LeuRS. We have extended this result by making a more drastic set of anticodon changes and concur that the $tRNA^{Leu}$ anticodon is not a recognition element. 2) The discriminator base; A73 which is conserved in all *E. coli* $tRNA^{Leu}$ sequences, is recognized by LeuRS. 3) The tertiary A15-U48 Levitt base pair may be an important recognition element since it is among the changes necessary to redirect the amino acid specificity of $tRNA^{Ser}$ to $tRNA^{Leu}$. 4) All *E. coli* $tRNA^{Leu}$ sequences have a characteristic dihydrouridine (D) loop which uniquely positions the conserved G18 and G19 bases relative to the D-stem. This positioning is also conserved in

$tRNA^{Ser}$ sequences but is different. The number of nucleotides preceding G18 G19 is one more in $tRNA^{Leu}$ than in $tRNA^{Ser}$ and the number of nucleotides following G18 G19 is one less. In the Ser-Leu redirection experiment, it was crucial to insert U15.1 into the $tRNA^{Ser}$ D-loop and delete A21. 5) The extra-stem loop in the $tRNA^{Leu}$ set is characterized by having one unpaired nucleotide between the base paired extra stem and the T-stem. In $tRNA^{Ser}$ there are no unpaired nucleotides between these two stems. While this extra-stem loop structure *per se* did not appear to be a recognition element, in the Ser-Leu change it was necessary to engineer an unpaired nucleotide between the two stems.

Despite this knowledge of the recognition elements of $tRNA^{Leu}$, it was not known which, if any of these elements were important *in vivo*, nor whether the changes affected only leucine identity. Changes to a tRNA could result in unanticipated acquisition of a different amino acid identity than the one intended or could fail (in recognition switch experiments) to remove the identity elements of the starting tRNA. However, with regards the likely non-participation of the extra stem-loop in $tRNA^{Leu}$ identity, there was agreement between *in vitro* and *in vivo* experiments. We had previously shown (90) that the type II extra loop in a $tRNA^{Leu}$ amber suppressor tRNA can be replaced by a consensus type I loop, with no loss of leucine specificity. For both serine and tyrosine amber suppressors, this alteration results in a switch in specificity to glutamine. From these results it appeared that the extra

stem loop was not a leucine identity element in agreement with the *in vitro* results of Asahara et al. (130).

That LeuRS does not recognize the extra stem loop was also indicated by alkylation protection experiments carried out by Dietrich et al. (131). In their experiments the interactions of $tRNA^{Leu}$ with the heterologous bean cytoplasmic LeuRS were studied by determining which phosphates are protected from alkylation by ethylnitrosourea. The results indicate that LeuRS interacts with $tRNA^{Leu}$ along the inside of the L-shaped tertiary structure on the opposite side from the extra stem loop.

Subsequently, the determination of the structures of a number of tRNA-aaRS complexes rationalize this result. Synthetases have been divided into two general groups on the basis of exclusive sets of amino acid sequence motifs (15, 89). Structural studies of tRNA-aaRS complexes have revealed that tRNAs interact differently with aaRS belonging to each group (9, 11, 13). Class I synthetases approach the L-shaped tRNA structure from the minor groove face of the acceptor stem. In contrast, most class II synthetases are expected to approach the tRNA from its variable loop side which allows for interactions with the major groove of the acceptor stem. LeuRS belongs to class I and is expected to interact with the D-loop side of the tRNA. SerRS belongs to class II and recognizes the base-paired variable stem loop as well as functional groups in the major groove of the acceptor stem helix (13, 28, 55, 65, 91).

In this study we have carried out an *in vivo* identity swap experiment to attempt the conversion of a $tRNA^{Ser}$ amber suppressor to leucine identity. In parallel, to understand further the role of the extra stem loop in $tRNA^{Leu}$, we have attempted to convert $tRNA^{Ser\Delta}$, an amber suppressor in which the type II extra stem loop had been substituted for a consensus type I loop, from its initial glutamine identity to leucine. *In vitro* aminoacylation assays for each of the tRNAs were performed using the competing synthetases, LeuRS, SerRS and GlnRS. While our results with the full length $tRNA^{Ser}$ to $tRNA^{Leu}$ swap support and extend the results of Asahara et al. (130), the results with the extra-stem loop deletion body ($tRNA^{Ser\Delta}$) reveal additional leucine identity elements that may play an important role in the complex *in vivo* competition of the 20 aaRS for $tRNA^{Leu}$.

MATERIALS AND METHODS

Materials

L-[3-^{3}H] leucine (S.A. 54 Ci/mmole), L-[3-^{3}H] serine (S.A. 30 Ci/mmole), and L-[3-^{3}H] glutamine (S.A. 46 Ci/mmole) were purchased from Amersham. DNA oligonucelotides were synthesized by the California Institute of Technology Biopolymer Synthesis Center on an Applied Biosystems DNA/RNA Synthesizer 394 (Perkin/Elmer). Native $tRNA^{Ser}$ and $tRNA^{Leu}$ were purchased from Subriden RNA. The restriction endonuclease BstN I and the plasmid pUC19 were purchased from New England Biolabs. T7 RNA polymerase was purified as previously described (144). Sequenase and sequencing

reagents were from U.S. Biochemical. Polyethyleneimine was from Miles Laboratories. Leupeptin and aprotinin were from Boehringer Mannhein Biochemicals. *E.coli* inorganic pyrophosphatase, bovine serum albumin, phenylmethylsulfonyl fluoride, benzamidine, pepstatin A, and methotrexate agarose were from Sigma. DEAE sephacel was from Pharmacia.

Strains and Plasmids

E. coli XAC-1 is *F'lacI373Zu118amproB^+/Δ(lacproB)x111nalA, rif, ara, argE_{am}.* Plasmid pGFIB-I and pDAYQ are described in (34, 36, 93).

Construction of tRNA genes

The genes for the STL variants and $tRNA^{Leu}\Delta_{CUA}$ were constructed by the ligation of six overlapping DNA oligonucleotides and were cloned into the EcoR I and Pst I sites of pGFIB-I. The genes for $tRNA^{Leu}_{CUA}$, $tRNA^{Ser}_{CUA}$ and $tRNA^{Ser}\Delta_{CUA}$ had been constructed previously by Normanly et al. (36, 90) and cloned into the EcoR I and Pst I sites of pGFIB-I. We used PCR to add a T7 RNA polymerase promoter and a BstN I restriction site to each of these genes and subsequently cloned them into the EcoR I and BamH I sites of pGFIB-I. Because these genes, as well as the STL variants, are active amber suppressors, we infer that neither the T7 polymerase promoter nor the BstN I site influence the correct processing of the tRNA in vivo. The genes for $tRNA^{Leu}_{UUU}$ and $tRNA^{Leu}_{UAA}$, which were constructed for use in only the in vitro experiments, were cloned into the EcoRI and Pst I sites of pUC19. The sequence of each tRNA gene was determined using the chain-termination method (Sequenase, USB).

Efficiency Assay

To determine the suppression efficiency of each amber suppressor variant, plasmids containing the suppressor tRNA were transformed into the *E. coli* lacIZ$_{am}$ fusion strain, XAC-1. The level of ß-galactosidase in this strain was compared to that of the isogenic wild-type strain XAC as described in (35).

Specificity Assay

The amino acid inserted by each tRNA variant was determined by constructing *E. coli* strains in which the tRNA variant and a reporter gene were co-expressed. In brief, a copy of the dihydrofolate reductase (DHFR) gene having an amber mutation at position 10 was expressed under the control of the tac promoter from the plasmid pDAYQ (36) and the tRNA variant was expressed from the compatible plasmid pGFIB-I. DHFR was expressed and purified as described below using a method described previously (36) with some modifications. Cells were grown in LB containing ampicillin (100 ug/ml) and chloramphenicol (30 ug/ml) to an optical density (A595) of 0.6. At this point the DHFR was induced by adding IPTG to a final concentration of 1 mM, and the cells were grown for 4 hours longer. The cells were centrifuged and the pellet was resuspended in buffer A (50 mM potassium phosphate pH 6.0, 200 mM KCl, 1 mM DTT, 1 mM EDTA, 100 µM PMSF). The resuspended cells were then sonicated three times (1 minute burst; 1 minute sitting on ice). The crude lysate was clarified by ultracentrifugation at 40K for 2 hours. The nucleic acids were removed from the clear lysate by precipitation with 1 % polyethyleneimine. The DHFR remained in the supernatant and it was loaded to a methotrexate

sepharose column, reloading the flow-through four times using a peristaltic pump . The column was then washed with buffer B (200 mM potassium borate pH 9.0, 1 M KCl, 1 mM DTT, 1 mM EDTA) until no more protein was detectable in the eluate. The DHFR was eluted with buffer C (200 mM potassium phosphate pH 8.0, 1 M KCl, 1 mM EDTA, 2 mM folic acid). The fractions containing DHFR were combined and dialyzed in buffer D (50 mM potassium phosphate pH 8.0, 1 mM DTT). The dialyzed sample was loaded on a DEAE-sephacel column, pre-equilibrated in buffer D. The column was developed with a gradient of 0-400 mM KCl in buffer D. The DHFR eluted at 250 mM KCl. The protein appeared homogeneous on a silver stained protein gel. The protein was dialyzed against water and was sequenced by Edman degradation at either the University of Southern California Microchemical Core Laboratory or the California Institute of Technology Protein/Peptide Micro Analytical Laboratory.

Purification of the Synthetases

E.coli LeuRS was purified from the *E.coli* MQ strain harboring the plasmid pLeuS 2 (101) leading to overproduction of LeuRS. Four liters of cells were grown at 37^0C to an optical density (A_{590}) of 0.8 in LB medium supplemented with 100 µg/ml of ampicillin. After inducing with 1 mM IPTG, the cells were grown to an absorbance of 1.7. Thirty-five grams of cells were harvested and resuspended in 60 ml of buffer A (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM ß-mercaptoethanol, and 25 mM KCl), sonicated and centrifuged at 100,000xg for 1 h at 4^0C. The protease inhibitors PMSF, leupeptin, pepstain A, benzamidine, and aprotinin were included throughout the

purification (104). Nucleic acid was removed from the lysate by precipitation with 1 % polyethyleneimine. LeuRS remained in the supernatant and was loaded to a 35 ml DEAE-sephacel column pre equlibrated with 10% glycerol Buffer A, and eluted with a gradient of 50-300 mM KCl in 10% glycerol Buffer A. LeuRS in the active fraction pool was precipitated with 75 % $(NH_4)_2SO_4$. The pellet was redissolved in 5 ml Buffer B (50 mM Tris-HCl pH 7.5, 1 mM DTT, and 10 % glycerol) and dialyzed into 50 % glycerol Buffer B and stored at -20^0C. The LeuRS appeared homogeneous on a Comassie Blue-stained protein gel. Silver staining of 1 μg of protein revealed few minor bands. The specific activity of the purified LeuRS was 2.3 x 10^5 units/mg protein. One unit is defined as the amount of LeuRS required to aminoacylate 1.0 nmole of $tRNA^{Leu}$ per minute at 37^0C at 5 μM $tRNA^{Leu}$ under the reaction conditions described in the aminoacylation reaction section. The protein concentration was established using the Bradford assay (BioRad) with bovine serum albumin as the standard, and was measured to be 40.6 mg/ml (360 μM). *E.coli* SerRS was purified as previously described (55) and GlnRS was purified following the protocol described by Söll and co-workers (97).

in Vitro Transcription of tRNAs

Plasmid DNAs were purified by equilibrium CsCl gradient centrifugation and were linearized by digestion with BstN I to obtain a transcription template allowing the synthesis of tRNAs with the proper 3'-terminal CCA (37). The transcription reactions contained : 40 mM Tris-HCl (pH 8.3 at 25^0C), 20 mM $MgCl_2$, 5 mM DTT, 1 mM

spermidine, 4 mM each NTP (pH 8.0), 10 mM GMP, 0.1 mg/ml BSA, 0.5 units/ml inorganic pyrophosphatase, 26,000 units/ml T7 RNA polymerase and 0.1 mg/ml DNA template. The reaction mixtures were incubated for 4 hours at 40°C, stopped by adding EDTA to a final concentration of 25 mM, phenol/chloroform extracted, ethanol precipitated, resuspended, and gel purified to single nucleotide resolution by electrophoresis under denaturing conditions (55). The native tRNAs were also gel purified. All tRNAs were eluted twice with 500 mM KOAc pH 5.4 at 4°C. The tRNAs were ethanol precipitated a total of four times, finally resuspended in 10 mM HEPES-KOH (pH 7.4) and stored at -20°C. RNA concentrations were quantitated by measuring the absorbance at 260 nm of a nuclease-digested sample (55). Extinction coefficients were calculated from the sequences of the tRNAs.

Aminoacylation Reactions

The LeuRS concentrations used to measure the kinetic parameters of various substrates ranged from 6.2 nM to 135 nM depending on the activity of the substrate. In a series of optimization experiments we determined that optimum aminoacylation by LeuRS could be obtained in reactions containing 30mM Hepes-KOH buffer, pH 7.4 and 50mM KCl. In particular, this buffer and salt combination gave aminoacylation rates that were two-fold greater than those obtained using 30 mM Tris-HCl (pH 7.5 at 25°C). Moreover, whereas the aminoacylation rates using HEPES were linear during a 15 minute reaction period, they began to decline in Tris-HCl, presumably due to the deacylation activity of this nucleophilic buffer (22). For consistency in comparing the activity of a particular substrate with different aaRSs we used the HEPES/KCl

combination for all three aaRSs employed in this study. All reaction mixtures were incubated at 37°C and contained 30 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 2 mM ATP, 20 μM amino acid (S.A. 10 ci/mmole). SerRS reactions were performed in the presence of inorganic pyrophosphatase (55).

All tRNAs were annealed by heating for three minutes at 80°C in 10 mM HEPES-KOH (pH7.4), 5 mM $MgCl_2$, and 0.05 mM EDTA (145) at a concentration 2.5 times greater than their final concentration in the aminoacylation reaction and allowed to slow cool to 25°C before use. For the kinetic experiments, the tRNA concentrations ranged from 0.2 to 1.6 mM and were always at least 10-fold greater than the enzyme concentration. A final protein concentration of 0.2 mg/ml in each reaction was obtained by adding BSA. At appropriate time points, aliquots were spotted on dried Whatman 3MM paper pretreated with 10% TCA and 100 μM amino acid. The spotted papers were washed two times with 10% TCA, three times with 5% TCA, and one time with ethanol for a period of time that reduced the residual background of ^{3}H-amino acid in a control reaction, not containing tRNA and independent of the aaRS concentration, to below 0.008% of the total radioactivity spotted. The counting efficiency of the ^{3}H-leucine on the Whatman filter paper in fluor (consisting of 3.83 grams of 2,5-diphenyloxazole per liter of toluene) was experimentally determined to be 25%.

RESULTS

Changing the Identity of a $tRNA^{Ser}$ to Leucine.

The sequences of the five leucine tRNAs from *E. coli* (anticodons CAG, GAG , UAG, CAA, UAA), and a $tRNA^{Leu}$ from bacteriophage T4 (anticodon UAA) were compared in order to detect specific residues that are likely to contribute to recognition by this synthetase. The T4 leucine tRNA is known to be aminoacylated by *E.coli* LeuRS (106). Figure 1 shows that there are 33 nucleotides which are conserved among these tRNAs. Of these, 14 occur in nearly all tRNAs and thus are not anticipated to differentially dictate recognition. This left 18 nucleotides that might either directly or indirectly dictate specific aminoacylation by LeuRS. Since our interest was in determining the nucleotide changes that were necessary to redirect the *in vivo* amino acid identity of a $tRNA^{Ser}$ to leucine, we excluded from consideration the 5 nucleotides that are found in both E. coli leucine and serine tRNAs as well as A35 of the anticodon. This left 12 nucleotides that differed between these two types of tRNAs and that might therefore account for their different amino acid identities under conditions where both synthetases are competing for the same substrate. Transplanting some or all of these nucleotides into $tRNA^{Ser}$ was therefore expected to be necessary for the acquisition of leucine identity. We additionally considered whether it was necessary to change other nucleotides in $tRNA^{Ser}$ in order to discourage aminoacylation by SerRS.

At the outset, it was necessary to determine the consequence of using amber suppressors for both the *in vivo* and in vitro assays. The anticodon is a crucial recognition element for most tRNAs (42, 51).

Thus the nucleotide changes that result when an amber (CUA) anticodon is transplanted into any tRNA can potentially redirect tRNA amino acid identity (33, 105) or, when the correct amino acid is inserted, the tRNA may nonetheless be impaired in aminoacylation efficiency with respect to the cognate synthetase (34, 58, 59). The existence of amber suppressing alleles of both $tRNA^{Leu}$ and $tRNA^{Ser}$ which retain their original amino acid identity showed that any change in aminoacylation efficiency by the cognate synthetase was not sufficient to redirect amino acid identity in the cell. Subsequent in vitro aminoacylation kinetic determinations using SerRS (55), as well as the $tRNA^{Ser}$-SerRS cocrystal structure (13) definitively showed that SerRS does not recognize nucleotides in this region of the tRNA. A similar situation was anticipated for $tRNA^{Leu}$ based on the existence of leucine inserting amber suppressors as well as from in vitro aminoacylation assays which showed that changing the $tRNA^{Leu}$ anticodon from CAG to UGA had no appreciable effect on aminoacylation by LeuRS (130). Nevertheless, the effect of an amber anticodon (CUA) on aminoacylation by LeuRS had not been directly determined.

Table 1 shows that changing the $tRNA^{Leu}$ anticodon from UAA to CUA decreased kcat/Km of aminoacylation by LeuRS by only about five fold. Due to the degeneracy of the genetic code, N36 of the leucine anticodon is normally a purine (either A36 or G36). Thus the possibility existed that neither the serine (UGA) nor the amber CUA anticodon affected aminoacylation by LeuRS because they present a required A36. This possibility was tested using a $tRNA^{Leu}$ variant having the anticodon UUU (lysine). As indicated in Table 1, this anticodon transplant had

only a trivial effect on aminoacylation by LeuRS. Thus *in vivo* and in vitro assays consistently indicate that E. coli LeuRS does not specifically recognize the anticodon of its cognate tRNAs. However, the anticodon is an important determinant of aminoacylation specificity for GlnRS (53, 146). So while the presence of the amber anticodon should not affect the competitive balance between LeuRS and SerRS, its presence in the tRNA variants described below is likely to alter the competitive balance by promoting aminoacylation by GlnRS.

Figure 2 shows the changes we selected for an initial Ser-Leu transformation (STL2). Many of the changes we have inserted are expected to affect the tertiary structure of the tRNA. These changes were made based on the tertiary interactions that are revealed in the crystal structures of $tRNA^{Phe}$ and $tRNA^{Asp}$ and the SerRS-$tRNA^{Ser}$ cocrystal structure (7, 8, 10, 13). The G15-C48 ("Levitt" tertiary base pair (147) of $tRNA^{Ser}$ was changed to A15-U48 for several reasons. An A15-U48 base pair is conserved among leucine tRNAs (Figure 1) and is commonly found within this isoaccepting group. Moreover, it is not found in other type II tRNAs. Finally, this base pair is expected to dictate tRNA tertiary structure and might thereby affect the positioning of other nucleotides that are directly recognized by LeuRS. The position of G18 and G19 within the D-loop differs among tRNAs could also contribute to subtle variations in the tRNA tertiary structure. In particular, G18 and G19 are typically preceded by three nucleotides and followed by four nucleotides in serine tRNAs but just the opposite configuration is found in leucine tRNAs. The $tRNA^{Leu}$ configuration was obtained by introducing an insertion and a deletion into the $tRNA^{Ser}$

D-loop. As a consequence of these changes, G20b of $tRNA^{Ser}$ potentially took on the role of the unusual G21 of leucine tRNAs. The C10-G25 base pair of the $tRNA^{Ser}$ D-stem was changed to G10-C25 and A59 was changed to G so that the nucleotides at these positions conformed to those conserved among leucine tRNAs. An unpaired nucleotide was inserted at position 47n of $tRNA^{Ser}$ between the extra stem-loop and the T-stem since this configuration is found in leucine but not serine tRNAs. In addition to introducing a portion of the conserved $tRNA^{Leu}$ sequence into $tRNA^{Ser}$, the insertion at position 47n, in conjunction with the deletion of A21, probably alters both the A21-[U8-A14] tertiary base pair as well as the base stacking interactions between G20b and the 45-47m base pair that normally occur in serine tRNAs (13). Consequently, it is likely that both the core structure of $tRNA^{Ser}$ as well as the orientation of the extra stem-loop were changed in a manner that discouraged aminoacylation by SerRS. STL2 was designed prior to the publication of the $tRNA^{Ser}$-SerRS cocrystal structure (13) and thus before the determinants of the extra stem-loop orientation in $tRNA^{Ser}$ were known. In the type I yeast $tRNA^{Phe}$, the G26-A44 "propeller" base pair stacks on G45 of the variable loop (7, 8). Thus the possibility existed that a similar type of base stacking interaction might affect the orientation of the extra stem-loop structure in $tRNA^{Ser}$. The A26G substitution was introduced in an attempt to alter this putative interaction. Finally, STL2 contains changes at position 73 and in the base pairs at positions 2-71 and 3-70 of the acceptor stem in order to recapitulate the sequence found in leucine tRNAs. Although the base pair at position 3-70 is not absolutely conserved among leucine tRNAs

(Figure 1), the G-C and C-G base pair alternatives at this position do conserve an exocyclic amino group in the minor groove. The change to C-G was suggested by a statistical study of $tRNA^{Leu}$ sequences which showed that the most commonly used leucine tRNA in *E. coli* contains a C3-G70 base pair (3).

We have carried out both *in vivo* and *in vitro* assessments of the activity of STL2 and a number of variants of this tRNA. Prior to the *in vitro* kinetic experiments we tested each of the STL substrates in preliminary aminoacylation experiments with LeuRS, SerRS and GlnRS to confirm that each tRNA could be completely charged, affirming that the transcript was not grossly mis-folded. Thus these experiments provide a positive control for substrates exhibiting poor kinetic constants for a particular aaRS (Figure 3).

The mutant STL2 is a good suppressor, 75% efficient as measured using the ß-galactosidase assay, and it exclusively inserts leucine. The in vitro kinetic experiments revealed a kcat/Km of aminoacylation by LeuRS that was only about 6 fold lower than that of the native $tRNA^{Leu}$. On the other hand, STL2 is an extremely poor substrate for SerRS (Table 1) indicating that the nucleotide changes have disrupted interactions with this synthetase. Sixteen changes are therefore sufficient to convert a $tRNA^{Ser}$ into a $tRNA^{Leu}$.

What remained to be determined was whether structural features, the acceptor stem sequence or both types of elements were necessary for the Ser-Leu transformation. The variant STL8 has the same structural alterations as STL2 but lacks the changes in the acceptor stem and at position 73. It is a good suppressor, 50% efficient as measured using the

ß-galactosidase assay. Although it inserts a substantial amount of leucine, it is additionally a substrate for GlnRS, HisRS and SerRS under *in vivo* conditions (Figure 2). The *in vitro* aminoacylation assays are consistent with this observation. STL8 had a kcat/Km of aminoacylation by LeuRS that was 120 fold lower than that of wild type $tRNA^{Leu}$. Although the *in vitro* aminoacylation assays showed that STL8 was an extremely poor substrate for SerRS, interestingly a small proportion of STL8 is aminoacylated with serine under *in vivo* conditions. Together, the results for STL8 indicate that the structural changes, alone, are not sufficient to redirect the amino acid identity of $tRNA^{Ser}$.

Two types of changes were introduced into the $tRNA^{Ser}$ acceptor stem in order to recapitulate the $tRNA^{Leu}$ acceptor stem sequence: changes in the acceptor stem and at position 73. STL7 lacks the acceptor stem changes but retains A73. Both STL2 and STL7 are very good suppressors and exclusively insert leucine under *in vivo* conditions. The in vitro aminoacylation assays revealed that LeuRS recognizes STL2 and STL7 with a similar efficiency; the kcat/Km of STL2 and STL7 was only 6.3 and 8.4 fold lower, respectively, than the wild-type substrate. Thus it was not necessary to change the base pairs of the helical acceptor stem in order to affect a Ser-Leu identity swap within the context of the structural changes that were introduced into the full length $tRNA^{Ser}$.

The second type of change in the acceptor stem involves the single-stranded "discriminator" nucleotide at position 73. E. coli serine tRNAs always have a guanosine at this position whereas in leucine tRNAs it is exclusively an adenine. The sequences of STL7 and STL8 only differ with respect to the nucleotide at position 73. STL7 and STL8

were good suppressors (50 and 80 percent, respectively). However, whereas STL7 exclusively inserts leucine, STL8 had a mixed amino acid identity (Figure 2). Interestingly, kcat/Km of aminoacylation by LeuRS differs between these variants by only a factor of about 14 fold. Thus A73 contributes to aminoacylation by LeuRS and its presence appears to affect the outcome of the competition among synthetases.

A second set of $tRNA^{Ser}$ variants was constructed to examine whether the nucleotides necessary to promote aminoacylation by LeuRS, both *in vivo* and *in vitro*, differ as a function of the structural framework in which they are embedded. Such an effect might be anticipated if the structural framework affects the orientation of recognition nucleotides in three-dimensional space and thus affects the accessibility of critical nucleotide functional groups (39). In addition, the structural framework could influence the stabilization of a transition state complex that is necessary for catalysis. Consequently, a change in the tRNA structural framework could not only affect recognition by the cognate synthetase but might also promote recognition by a non-cognate synthetase affecting the set of synthetases which are competing for a given substrate.

We had previously engineered a variant, $tRNA^{Leu\Delta}$, in which the type II extra stem-loop of a $tRNA^{Leu}$ amber suppressor was replaced by a consensus type I loop with no loss of leucine specificity (90). This result suggested that the $tRNA^{Leu}$ extra stem-loop structure is a dispensable element in vivo and unlikely to be in direct contact with LeuRS, a conclusion that was consistent with the results of footprinting studies of leucine tRNAs and LeuRS (131). Although LeuRS does not

directly contact the extra stem-loop, changes in this region of the tRNA were not without effect; the kcat/Km of aminoacylation by LeuRS is reduced by about 40 fold when the extra stem-loop of $tRNA^{Leu}_{CUA}$ is replaced with the type I consensus loop to give $tRNA^{Leu\Delta}_{CUA}$ (Figure 4 and Table 1).

In contrast, the $tRNA^{Ser}$-SerRS cocrystal structure (13) shows that SerRS directly contacts the $tRNA^{Ser}$ extra stem-loop. Moreover, both in vivo (90) and in vitro (55, 137, 138) assays show that this structural feature makes a large contribution to serine specificity. Interestingly, when the type II extra loop in a $tRNA^{Ser}$ amber suppressor is replaced with a consensus type I loop to create $tRNA^{Ser\Delta}$, the variant exclusively inserts glutamine, rather than serine, in the in vivo DHFR assay (90). Thus $tRNA^{Ser\Delta}$ provides an interesting scaffold for examining the requirements for leucine identity. Importantly, even though $tRNA^{Ser}$ and $tRNA^{Ser\Delta}$ only differ with respect to nucleotides in the region of the extra loop, they are substrates for two different synthetases.

The variant STL3 is analogous to STL2. It has the full complement of nucleotide changes seen in STL2 but instead of the type II extra stem-loop, STL3 has a consensus type I variable loop. Whereas the *in vivo* assay showed that STL2 inserted only leucine, STL3 predominantly inserts leucine (93%) and a small amount of glutamine (7%). Thus the same set of nucleotide changes in the STL2 and STL3 structural frameworks had a slightly different outcome. The results of the *in vitro* aminoacylation assays are consistent with these observations:

the kcat/Km of aminoacylation by LeuRS of STL2 is about 4 fold greater than that of STL3, primarily due to a difference in Km.

The variant STL5 has the same structural alterations as STL3, but lacks the changes in the helical acceptor stem and at position 73. Although both tRNAs are efficient suppressors, they differ in amino acid identity. STL3, the variant having the $tRNA^{Leu}$ acceptor stem sequence, inserts leucine in the DHFR assay, whereas STL5 which lacks these changes exclusively inserts glutamine. The *in vitro* aminoacylation assays showed that STL5 is an extremely poor substrate for LeuRS (Table 1). The possibility that the tRNA is improperly folded is ruled out by the fact that STL5 can be aminoacylated to nearly 100% by GlnRS (Figure 3). It is striking that removing the $tRNA^{Leu}$ acceptor stem discriminator base sequences from the full length tRNA scaffold (STL2 vs STL8) decreased kcat/Km of aminoacylation by LeuRS by 120 fold compared to a wild type $tRNA^{Leu}$ while their lack in the $tRNA^{Ser\Delta}$ scaffold (STL3 vs STL5) rendered STL5 unchargeable by LeuRS (Table 1).

Comparison of the data for STL3 and STL6 indicates that the sequence of the helical acceptor stem plays a role in dictating tRNA amino acid identity within the structural scaffold lacking the extra stem-loop. This is reflected by the results of the assay which reveal that STL3 inserts 93% leucine and 7% glutamine while STL6 only inserts 66% leucine and 34% glutamine. The *in vitro* aminoacylation assays show a similar trend. The kcat/Km of aminoacylation by LeuRS of STL3 is 23 fold lower than that of a wild type $tRNA^{Leu}$ whereas the kcat/Km of STL6 is 3000 fold lower. However the results for STL3 and STL6 are

striking in that a large difference in the *in vitro* aminoacylation kinetics of these two tRNAs with respect to LeuRS results in a rather modest difference in their amino acid specificities within the cellular environment.

The effect of A73 on recognition by LeuRS is revealed by comparing the data for STL5 and STL6 whose sequences only differ with respect to the nucleotide at position 73. STL5, having a G73, inserts 100% glutamine whereas STL6, having an A73 inserts only 34% glutamine and 66% leucine. Thus the G73A substitution, which introduces the conserved A73 of *E.coli* leucine tRNAs, markedly increases the amount of leucine that is inserted into the reporter protein. This identity change seems to stem, in part, from an improvement in the aminoacylation efficiency by LeuRS since the kcat/Km of STL6 (A73) and STL5 (G73) differ by a factor of at least 4. Nevertheless, STL6 is a poor substrate for LeuRS having a kcat/Km of aminoacylation that is 3000 fold lower than that of a wild-type $tRNA^{Leu}$ (Table 1 and Figure 2). STL6 can be aminoacylated to 80% when high enzyme concentrations and long time periods are used (Figure 3), ruling out the possibility that it is grossly misfolded. Thus although an A73 in the $tRNA^{\Delta}$ framework contributes to recognition by LeuRS, it is not sufficient to entirely confer leucine identity *in vivo* nor to confer efficient aminoacylation by LeuRS.

The difference in the amino acid identities of STL5 and STL6 appears to stem from an influence of the nucleotide at position 73 on recognition by GlnRS as well as LeuRS. Whereas nearly 100% of the input STL5 was aminoacylated in ten minutes using an excess of GlnRS,

only about 10% of STL6 was aminoacylated in this time period using the same enzyme and substrate concentrations (Figure 3). Given the conditions used for these experiments, it is not possible to ascertain absolute differences in the aminoacylation efficiencies of these two substrates. Nevertheless, it is clear that there is a qualitative improvement in aminoacylation by GlnRS when the G73 (STL5) as opposed to the A73 (STL6) is present. Moreover, it is well established that G73 in the wild-type $tRNA^{Gln}$ is a recognition element for GlnRS (11, 53).

In the variant STL9 (Figure 5) we attempted to obtain the Ser-Leu transformation utilizing a minimum number of changes: the G73A change, nucleotide substitutions which presumably altered the orientation of the extra stem-loop, and the introduction of the A15-U48 "Levitt" base pair. STL9 is only 10% efficient, as measured by the b-galactosidase assay but it inserts 100% leucine. Its kcat/Km of aminoacylation by LeuRS was only about 8 fold lower than that of a wild-type $tRNA^{Leu}$. Moreover, it was a very poor substrate for SerRS having a kcat/Km that was 2300 fold lower than that of a wild-type $tRNA^{Ser}$. Thus only six changes are required to transform $tRNA^{Ser}$ into a tRNA that accepts only leucine in the cell and that has near wild type activity with respect to LeuRS.

In an attempt to delineate the necessity of these changes, we designed two additional variants. In STL12 the "Levitt" base pair is reverted to the sequence that is normally found in serine tRNAs but all other changes are preserved. This variant is a rather poor suppressor (1% efficient) and is not aminoacylated with leucine in the cell. Rather,

it has an amino acid identity that is 95% serine and 5% tyrosine. This indicates that the A15-U48 "Levitt" base pair characteristic of leucine tRNAs is critical to their amino acid identity. The A15-U48 "Levitt" base pair is retained in STL13 as is the G73A change. However, in this variant the D-loop and extra stem-loop sequences are reverted to those of serine tRNAs. STL13 has an efficiency of 40% but it is not aminoacylated with leucine in the cell. Rather, it inserts 97% serine and 3% tyrosine. This indicates that the A15-U48 "Levitt" base pair and A73 are not sufficient to ensure leucine identity. Thus the *in vivo* results show that the D-loop, extra stem-loop and the G73A substitutions (STL9) are all required for the complete transformation of a $tRNA^{Ser}$ into a $tRNA^{Leu}$ under conditions where all 20 synthetases are competing for substrates. Asahara and coworkers (130) showed that kcat/Km of aminoacylation by LeuRS was decreased by about 280 fold when the A15-U48 base pair was reverted to G15-C48 (as in STL12). In addition, kcat/Km of aminoacylation by LeuRS was below detectable limits for a variant having an A73 and the A15-U48 base pair but in which the D-loop and extra stem-loop were in the $tRNA^{Ser}$ configuration. Thus the *in vitro* and *in vivo* results for these minimal substrates are in agreement.

DISCUSSION

The nucleotides which determine tRNA amino acid specificity can be identified using either an *in vitro* or an *in vivo* approach. *In vitro* studies typically focus on the determinants of cognate interactions. The

results obtained using the *in vivo* approach reflect the outcome of the competition between the cognate aaRS and any unfortuitous interactions between the tRNA and non-cognate synthetases. In our earlier *in vivo* study of $tRNA^{Ser}$ identity we showed that eight changes were necessary to convert a $tRNA^{Leu}$ amber suppressor to serine identity (36, 90). Subsequent *in vitro* studies elucidated the recognition elements of $tRNA^{Ser}$ (55, 65, 137, 138). In this paper we examine the determinants of amino acid specificity for *E. coli* $tRNA^{Leu}$ by attempting the opposite transformation: redirecting the specificity of a $tRNA^{Ser}$ amber suppressor to leucine identity. Both $tRNA^{Ser}$ and $tRNA^{Leu}$ are type II tRNAs containing a large extra stem loop. In addition to transforming $tRNA^{Ser}$ to $tRNA^{Leu}$, a "type II swap," we attempted a "type I swap," the transformation of amber suppressor $tRNA^{Ser\Delta}$ to leucine identity. $tRNA^{Ser\Delta}$ was constructed earlier to explore the role of the type II stem loop structure in serine identity (55, 90) In this tRNA the base paired extra stem-loop common to type II tRNAs is replaced by a consensus 5 base extra loop characteristic of type I tRNAs. This type I'amber suppressor inserts glutamine so this is a Gln-Leu swap. For each of the tRNA variants we determined both the *in vivo* identity and also determined the *in vitro* specificity for aminoacylation by LeuRS, SerRS and GlnRS. This combination of both *in vivo* and *in vitro* methods is useful because each gives a different perspective on the complex interactions that determine tRNA identity in the cell.

For our *in vivo* approach, each tRNA variant must have the CUA amber anticodon. This can influence the experimental outcome in two ways. In cases where the cognate synthetase recognizes the anticodon,

nucleotide substitutions creating the amber (CUA) anticodon can weaken that interaction (34, 58, 59) and might thereby facilitate the acquisition of a new identity when additional changes are made. The amber anticodon can also directly promote interactions with a non-cognate synthetase changing the identity of the tRNA (33, 105). Since neither SerRS (13, 55) nor LeuRS ((130) and Table 1) recognizes the anticodon of the cognate tRNA, the $tRNA^{Ser}$ to $tRNA^{Leu}$ transformation is ideal for this approach. Nevertheless, because the amber anticodon provides recognition elements for GlnRS (23, 53, 146), introduction of the amber anticodon was not entirely without consequence. Indeed, competition between SerRS, LeuRS and GlnRS for the amber suppressor tRNA is revealed by the *in vivo* data (Figures 2 and 5). Importantly, $tRNA^{Ser\Delta}$ has glutamine identity (90). Thus the identity swap being attempted in this case is from glutamine to leucine.

In the type II swap, six changes, five affecting the tertiary structure and one in the discriminator base, were necessary and sufficient to completely change the identity of the full length $tRNA^{Ser}$ amber suppressor to leucine. The tertiary changes involved the configuration of the D-loop, the 3'-end of the extra stem-loop, and the trans-tertiary A15-U48 "Levitt" base pair. (STL9; Figure 5).

a). The discriminator base.

The variants STL7 and STL8 have the same nucleotide changes embedded in a full length $tRNA^{Ser}$ scaffold and differ only in position 73. The *in vivo* assays reveal that if the discriminator base is G, as in STL8, the variant inserts 66% leucine but it also inserts glutamine, histidine and serine. However, when the discriminator base is changed

to A, the variant inserts only leucine. The *in vitro* aminoacylation assays revealed that STL8 (G73) has a kcat/Km of aminoacylation by LeuRS that is about 14 fold lower than that of STL7 (A73). The same substitution in a $tRNA^{Leu}$ reduces V_{max}/K_m by about 160-fold (130). In contrast, aminoacylation by SerRS and GlnRS was relatively unaffected by the nucleotide at position 73 (Figure 3 and Table 1). Thus a positive effect of A73 on recognition by LeuRS appears to account for the different amino acid identities of STL7 and STL8.

It was not possible to estimate the individual kinetic parameters or STL8 for LeuRS using the tRNA concentrations employed in this study. Nevertheless, it appears that the relatively low specificity constant of STL8 is due, in part to an elevated Km. We estimate this value to be no less than 15μM since at this concentration the initial velocity was proportional to the substrate concentration. An effect of the discriminator base on Km is somewhat unexpected given the close proximity of this nucleotide to the synthetase active site at the time of amino acid transfer. However, an elevated Km has also been reported for a G73A substitution in a $tRNA^{Leu}$ (130). Moreover, an elevated Km is consistent with the *in vivo* results. There was little, if any, difference in the aminoacylation specificity of STL8 and STL7 for GlnRS and SerRS; yet STL8 had a mixed amino acid identity whereas STL7 did not. It is possible that as a consequence of the relatively high Km of STL8 for LeuRS, the concentration of free STL8 in the cell was elevated to an extent that allowed GlnRS and SerRS to effectively compete for this substrate. Indeed, tight binding among cognate pairs

can be an essential determinant of tRNA amino acid identity (4, 142) as can the tRNA and synthetase concentrations (24, 44, 141, 143).

b). The A15-U48 tertiary base-pair.

The contribution of the A15-U48 tertiary base pair to leucine idenity was revealed by comparing the amino acid identity of STL9 to that of STL12 (Figure 5). Both STL9 and STL12 have the $tRNA^{Leu}$ A73, D-loop configuration and the unpaired nucleotide at the 3'-end of the extra stem-loop. However, whereas STL9 has the $tRNA^{Leu}$ A15-U48 base pair, STL12 has the G15-C48 base pair of $tRNA^{Ser}$. STL9 inserted only leucine, but STL12 inserted 95% serine and 5% tyrosine. Thus an A15-U48 base pair is essential for leucine identity. The *in vitro* studies of Asahara et al. (130) confirm that this base pair is an important recognition element. As we shall see below it is likely to be the trans-tertiary interactions that this base-pair participates in trans-tertiary interactions that are important for its role in leucine identity.

c). The D-loop configuration and the orientation on the extra stem-loop .

All *E.coli* leucine tRNAs have a characteristic D-loop in which there are three unpaired nucleotides before the conserved G18 G19 and two after. In contrast, all serine tRNAs have the opposite configuration, two before and three after. These features are believed to contribute unique characteristics to the tertiary structure of the tRNA through their effects on positioning G18 and G19 which form tertiary base-pairs with Y55 and C56 in the T-loop (7, 8, 10). In addition all leucine tRNAs have a single unpaired base between the type II base-paired extra stem and the T stem. In *E. coli* serine tRNAs there are none. The

characteristic conformation of the D-loop and the nucleotides at the base of the extra stem are linked features, at least in $tRNA^{Ser}$, because there is a stacking interaction between the 45-47q base-pair and G20b that cannot take place in $tRNA^{Leu}$ where neither the base-pair nor G20b exist (13). Moreover in this context, the G15-C48 base-pair in $tRNA^{Ser}$ interacts with the dihydrouridine (D) at position 20a to form a base triple which influences the tRNA tertiary fold (13), an unlikely interaction in $tRNA^{Leu}$. These tertiary interactions are doubtless interrupted in the successful STL constructs although what replaces them or what their counterparts might be in $tRNA^{Leu}$ will not be known until we have a $tRNA^{Leu}$ structure.

The contributions of the D-loop and extra stem-loop features to leucine identity were revealed by comparing the amino acid identity of STL9 to that of STL13. Both STL9 and STL13 have the leucine A73 and A15-U48 tertiary base pair. And while STL9 has the $tRNA^{Leu}$ configuration in the D-loop and extra stem-loop, in STL13 the configuration is reverted to the one found in $tRNA^{Ser}$. STL9 inserted only leucine, but STL13 inserted 97% serine and 3% tyrosine. Thus A15-U48 cannot confer identity in the absence of a characteristic D-loop/extra stem loop configuration. It is striking that most of the important identity elements in $tRNA^{Leu}$ involve linked determinants of its tertiary structure.

It seems likely that the different D-loop and extra stem-loop configurations of STL9 and STL13 concomitantly affect recognition by both LeuRS and SerRS, but in opposing ways. STL9 is aminoacylated by LeuRS with an efficiency that is only about 8 fold lower than that of a

wild type $tRNA^{Leu}$ whereas it is aminoacylated by SerRS with an efficiency that is more than 2000 fold lower than that of tRNASer (Table 1). Although aminoacylation kinetics were not determined for STL13, it seems likely that it has just the opposite properties of STL9. STL13 is expected to be a poor substrate for LeuRS based on the results of Asahara and coworkers (130) who reported that V_{max}/K_m of aminoacylation by LeuRS of a tRNA variant having a sequence similar to that of STL13 was more than 5000-fold lower than a substrate resembling STL9. In contrast, STL13 is expected to be a good substrate for SerRS because it retains the 45-479 base pair and the location of the $tRNA^{Ser}$ G20b which affects the orientation of the extra stem-loop structure contributing to $tRNA^{Ser}$ recognition (13).

Based on the above observations, it appears that some of the determinants for recognition by SerRS and LeuRS reside in the same region. However, the mechanisms that account for the effect of these nucleotides on recognition by the cognate appear to differ. SerRS directly contacts the extra stem-loop structure (13). Thus nucleotide changes that alter the orientation of this structure are expected to affect aminoacylation by SerRS. In contrast, a direct contact between LeuRS and the extra stem-loop seems unlikely. We have shown that the $tRNA^{Leu}$ extra stem-loop can be replaced with the consensus type I variable loop with no loss of leucine identity (90) and that this change decreases kcat/Km of aminoacylation by LeuRS by only about 40 fold (Table 1; cf. LeuCUA and LeuΔCUA). Moreover, the results of alkylation protection experiments using *E. coli* $tRNA^{Leu}$ and the heterologous bean cytoplasmic LeuRS revealed no protection of this

structure (131). Instead, the experiments revealed protection on the 5'- and 3'-sides of the D-stem indicating that LeuRS binds the inside of the L-shaped tRNA structure. This binding topology, perhaps generating a different angle between the two helical arms of the tRNA or subtly different conformations of the backbone along the inside of the "L", provides a possible means by which LeuRS can discriminate between the subtly different tRNA tertiary structures in $tRNA^{Leu}$ and $tRNA^{Ser}$.

d) The Type I swap: $tRNA^{Ser\Delta}$-$tRNA^{Leu}$

In a second set of variants, the tRNA structural framework was changed by replacing the extra stem-loop of $tRNA^{Ser}$ with the consensus variable loop of type I tRNAs to give $tRNA^{Ser\Delta}$. This changed the set of synthetases that were competing with LeuRS as well as the structural framework within which the acceptor stem and discriminator base were presented to LeuRS. $tRNA^{Ser\Delta}$ has glutamine amino acid identity, in vivo, (90). When the $tRNA^{Leu}$ D-loop configuration and A15-U48 base pair are embedded within this framework to give STL5, the amino acid identity is still glutamine. The *in vitro* aminoacylation assays revealed that STL5 is an extremely poor substrate for LeuRS but that it is aminoacylated to nearly 100% by glutamine synthetase (Figure 3 and Table 1), ruling out the possiblity that the variant is incorrectly folded. Comparison of the data for STL5 and STL6 reveals the influence of A73 in the $tRNA^{Ser\Delta}$ framework. Whereas STL5 had an *in vivo* amino acid identity that was completely glutamine, STL6 inserts 34% glutamine and 66% leucine. The *in vitro* aminoacylation assays revealed that aminoacylation of STL5 by LeuRS was below detectable limits but is improved somewhat by the introduction of A73 (Table 1). While A73

was sufficient to completely confer STL7 with a leucine identity in the type II swap, it improved but did not completely confer leucine identity in the type II experiment. Comparison of the data for STL6 and STL3 reveals the influence of the $tRNA^{Leu}$ acceptor stem sequence. Substitutions in this region of the tRNA account for an additional increase in leucine identity, and a substantial increase in aminoacylation efficiency by LeuRS, rendering STL3 a good though not perfect leucine suppressor since it still inserts 7% glutamine (Table 1 and Figure 2). In contrast, the acceptor stem changes were not necessary within the full length tRNA background (cf. STL7 and STL2; Figure 2).

Two factors appear to account for our ability to achieve a complete identity swap in the type II body while not accomplishing it completely in the type I body. GlnRS competes poorly with LeuRS and SerRS for the full length variants even though each of these has an amber (CUA) anticodon which provides recognition elements for GlnRS. Thus the Ser-Leu transformation primarily depended on substitutions which depressed aminoacylation by SerRS and increased aminoacylation by LeuRS. Since some of the determinants for recognition by SerRS and LeuRS reside in the same region of the tRNA, it was possible to introduce nucleotide substitutions which had opposing effects on these two synthetases. In contrast, GlnRS is able to compete effectively with LeuRS and SerRS for the $tRNA^{Ser\Delta}$ variants. This is evidenced by the fact that $tRNA^{Ser\Delta}$ has a glutamine identity. Thus the acquisition of a leucine identity in the $tRNA^{\Delta}$ background necessitated substitutions that increased aminoacylation by LeuRS and that decreased aminoacylation by GlnRS. The *in vitro* assays show that increased aminoacylation by

LeuRS was achieved by introducing all of the conserved tRNALeu nucleotides into the $tRNA^{Ser\Delta}$ background (STL3; Figure 2). However, these substitutions did not compeletely suppress aminoacylation by GlnRS since STL3 still accepts some glutamine. Whereas the consensus leucine acceptor stem is expected to decrease aminoacylation specificity of the tRNA for GlnRS since it lacks some of the $tRNA^{Gln}$ recognition elements (11, 52, 53), STL3 retains recognition elements for GlnRS in its anticodon. Thus a complete identity swap was hampered by the different locations of recognition elements for LeuRS and GlnRS.

Two potentially important differences in the tertiary structures of the type II and type I variants may be a second, and related factor, influencing the success of the identity swap experiments. First, the $tRNA^{\Delta}$ variants have a single-stranded uridine rather than guanosine at the 3'-end of the variable loop. The results of Asahara et al. (130) when compared to ours indicate that the nucleotide at this position does not play a significant role in dictating recognition by LeuRS (our constructs have a G in that position, theirs have a U) However, it remains unclear whether U47 in the $tRNA^{Ser\Delta}$ framework and G47n in the full length tRNA backgrounds are in functionally equivalent positions. This could be critical if N47n plays a role in dictating the tertiary structure of $tRNA^{Leu}$. Second, in replacing the extra stem-loop with the type I consensus sequence, we may have inadvertently affected base triples so that the full length and $tRNA^{Ser\Delta}$ variants have subtly different tertiary structures.

In the type II tRNA framework, it was not necessary to introduce changes into the helical acceptor stem in order to affect a Ser-Leu

identity swap. In addition, nucleotide substitutions in the acceptor stem resulted in only a minimal improvement in aminoacylation by LeuRS (Figure 2 and Table 1). In contrast, in the type I framework, the acceptor stem substitutions were necessary for leucine identity and for efficient aminoacylation by LeuRS (Figure 2 and Table 1). This raises the possibility that LeuRS contacts the acceptor stem helix in a base-specific manner. Perhaps subtle differences in the tertiary structures of the type II and type I variants resulted in different orientations of the acceptor stems in the LeuRS active site allowing the contributions of these nucleotides to aminoacylation by LeuRS to be revealed. Interestingly we have observed a similar phenomenon in our study of $tRNA^{Ser}$ identity. Changes to the conserved acceptor base-pair, R4-Y69 were deleterious in impaired $tRNA^{Ser}$ backgrounds, e.g. in minihelices or in tRNAs lacking the extra stem loop but the same changes had little effect in full length $tRNA^{Ser}$ (65). A contribution of this base pair to recognition by SerRS is consistent with specific RNA-protein interactions in this region seen in the refined structure of the homologous *T. thermophilus* $tRNA^{Ser}$-SerRS complex (28). In light of the results for the serine system, and the observed "body specific" effects of the acceptor stem sequence on aminoacylation by LeuRS, it seems possible that LeuRS does in fact recognize the leucine specific bases in the acceptor stem.

Figure 1. Representation on a typical cloverleaf secondary structure of the residues absolutely conserved among the five*E.coli* $tRNA^{Leu}$ species and the one coded by bacteriophage T4. The indicated nucleotides are divided in three different classes: 1) residues invariant among all tRNAs (letters in bold characters); 2) residues conserved only among $tRNA^{Leu}$ (in outlined letters); 3) residues conserved among $tRNA^{Leu}$ that differ from $tRNA^{Ser}_{CGA}$ (shaded outlined letters). G21 is not conserved in T4 $tRNA^{Leu}$ but is found in all *E.coli* $tRNA^{Leu}$ and in most bacterial $tRNA^{Leu}$.

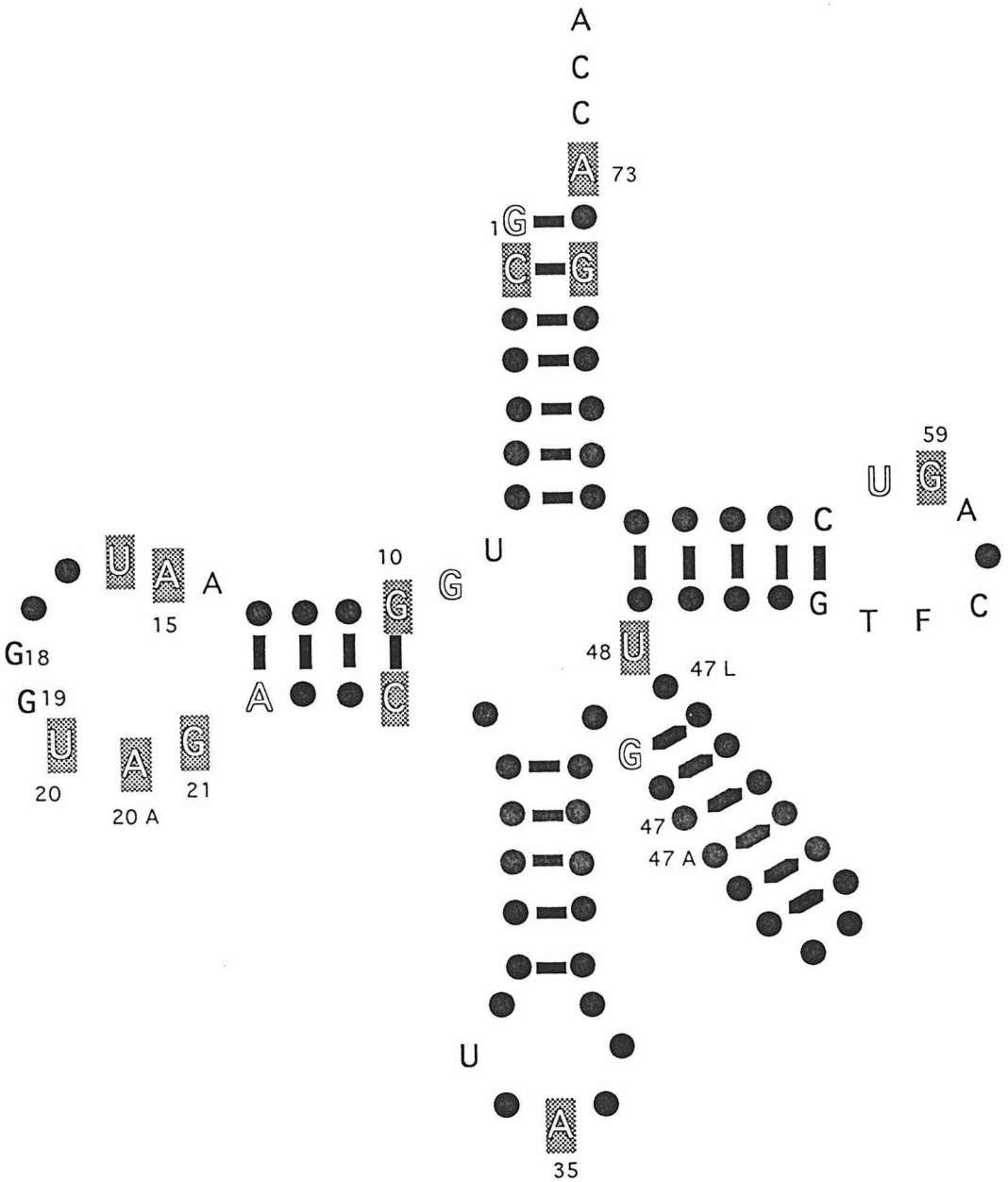
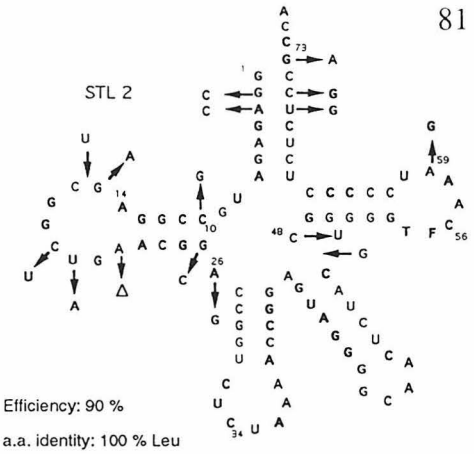

A
C
C
A 73
1 G
C G
59
U G A
C
U
10
G
U A A
15
G18
G19
U
20
A
20 A
G
21
A
G
C
T F C
48 U
47 L
G
47
47 A
U
A
35

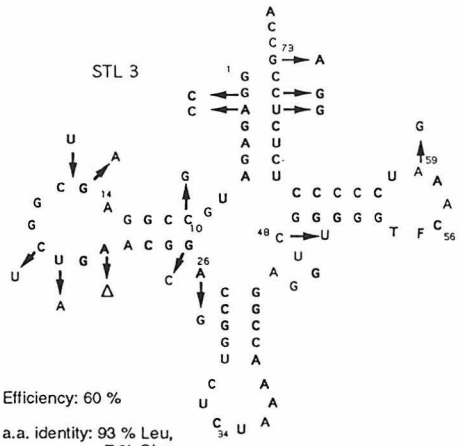
Figure 2 Sequences of STL variants. Efficiencies and identities were determined as described in the materials and methods section. LeuRS specificity changes indicate the level of decrease in K_{cat}/K_M relative to that of a T7-transcribed wild type tRNA. See Table 1 and Materials and Methods for experimental details. n.m.= not measurable.



Efficiency: 90 %

a.a. identity: 100 % Leu

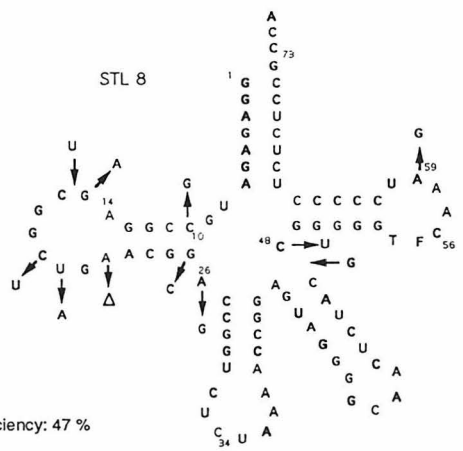
LeuRS specificity loss: 6.3



Efficiency: 60 %

a.a. identity: 93 % Leu,
7 % Gln

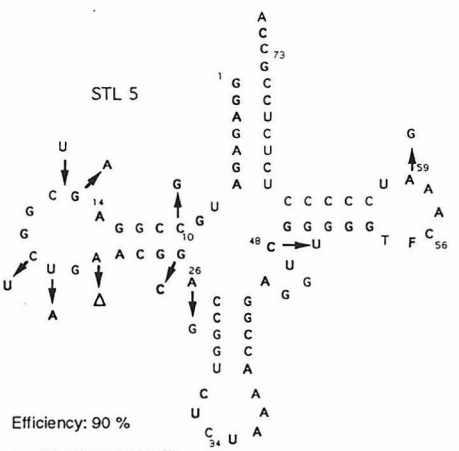
LeuRS specificity loss: 23.4



Efficiency: 47 %

a.a. identity: 66 % Leu, 13 % Gln,
6.5 % His, 4 % Ser

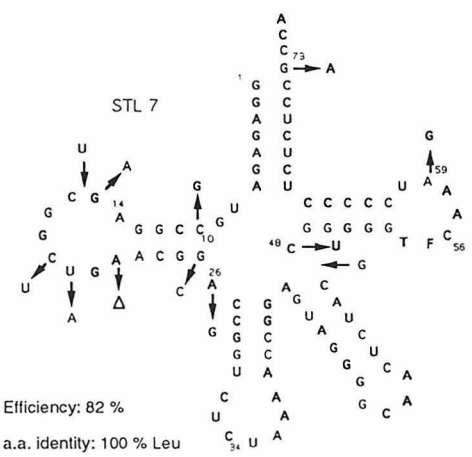
LeuRS specificity loss: 117



Efficiency: 90 %

a.a. identity: 100 % Gln

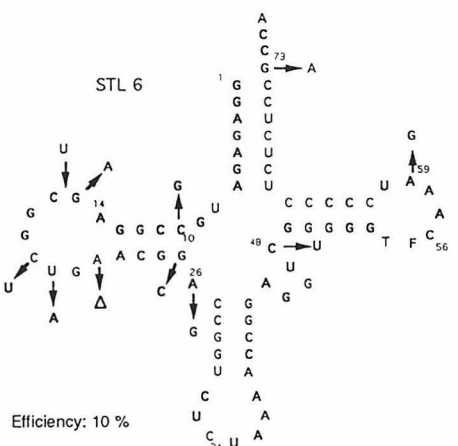
LeuRS specificity loss: > 12789



Efficiency: 82 %

a.a. identity: 100 % Leu

LeuRS specificity loss: 8.4



Efficiency: 10 %

a.a. identity: 66 % Leu,
34 % Gln

LeuRS specificity loss: 2957

Figure 3 Aminoacylation plateau levels were used to assess whether a variant tRNA maintained a tRNA-like structure, and was functionally active. Only variant transcripts that could be fully charged by at least one of the three aaRS were considered for aminoacylation kinetic studies (see Table 1). All reactions were performed at 1 μM tRNA and 2 μM aaRS under the conditions described in Materials and Methods.

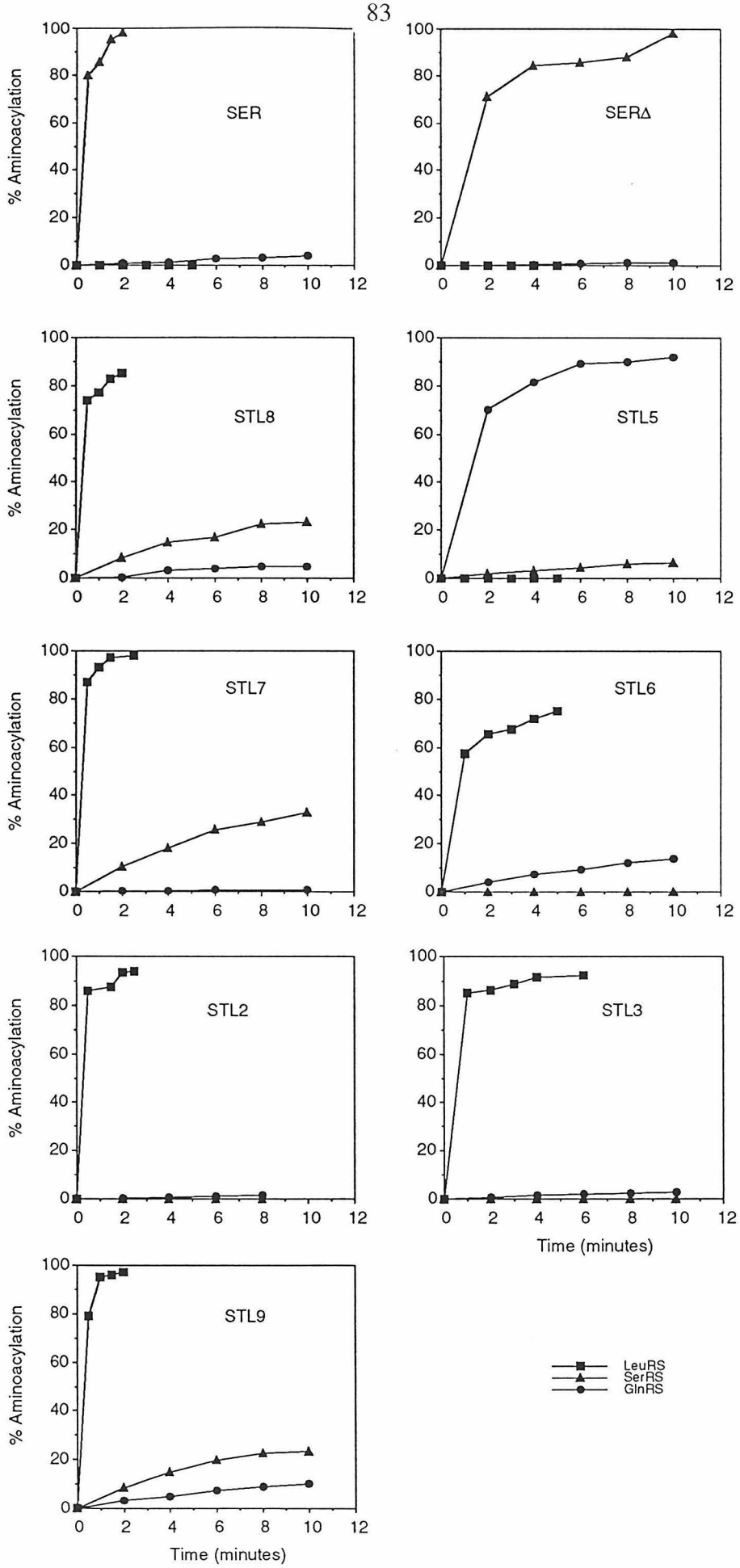
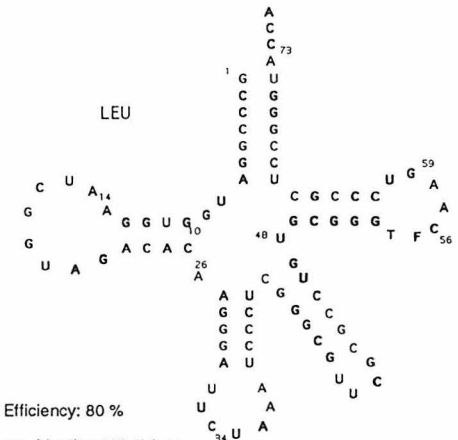

SER
SERΔ
STL8
STL5
STL7
STL6
STL2
STL3
STL9
% Aminoacylation
Time (minutes)
LeuRS
SerRS
GlnRS

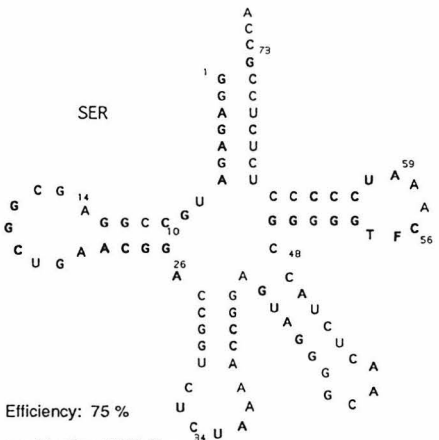
Figure 4 Sequences of $tRNA^{Ser}$ and $tRNA^{Leu}$ amber suppressors and extra-loop replacement variants. Suppression efficiencies and the amino acid identities were determined by (Normanly et al., 1986a; Normanly et al., 1992). In the Normanly et al. identity experiments $tRNA^{Leu\Delta}$ had a C at position 48.



Efficiency: 80 %

a.a. identity: 100 % Leu

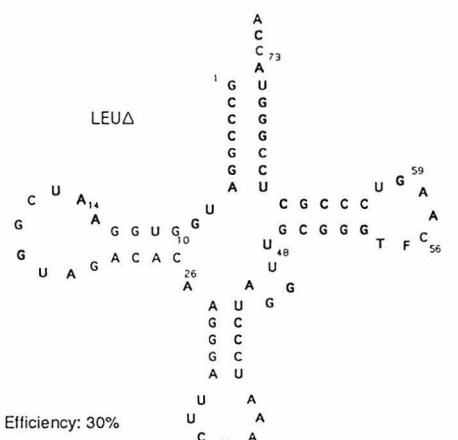
LeuRS specificity loss: (1)



Efficiency: 75 %

a.a. identity: 92 % Ser

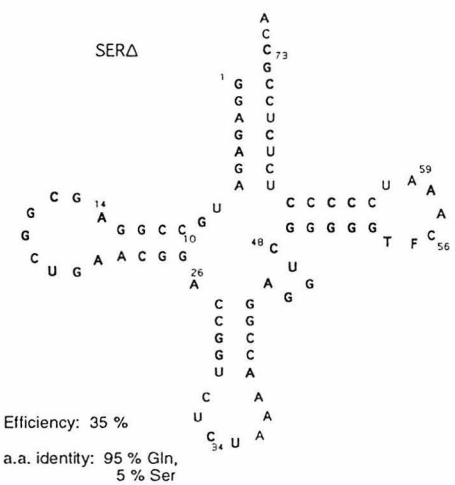
SerRS specificity loss: (1)



Efficiency: 30%

a.a. identity: 100 % Leu

LeuRS specificity loss: 200



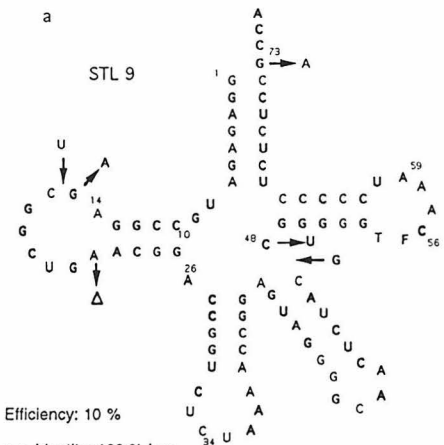
Efficiency: 35 %

a.a. identity: 95 % Gln,
5 % Ser

SerRS specificity loss: 4551

Figure 5 (a) $tRNA^{STL9}$, a $tRNA^{Ser}$ variant with the minimal changes that confer $tRNA^{Leu}$ identity. (b) The $tRNA^{STL9}$ variants STL12 and STL13. n.d.= not determined

a

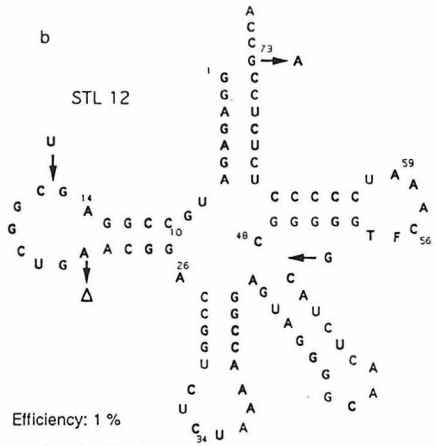


Efficiency: 10 %

a.a. identity: 100 % Leu

LeuRS specificity loss: 8.4

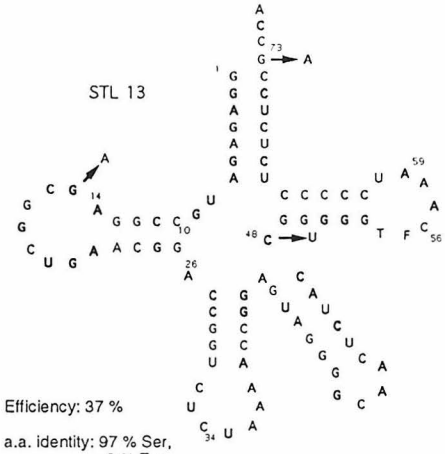
b



Efficiency: 1 %

a.a. identity: 95 % Ser,
5 % Tyr

LeuRS specificity loss: n.d.



Efficiency: 37 %

a.a. identity: 97 % Ser,
3 % Tyr

LeuRS specificity loss: n.d.

Table 1 Kinetic parameters for the aminoacylation of tRNA variants by LeuRS and SerRS of the tRNA studied.

The kinetic parameters for $tRNA^{LeuUAA}$, CUA, UUU, STL2, STL3, STL7, and STL9 were determined using at least five RNA concentrations ranging from 0.2 μM to 1.6 μM at a LeuRS concentration of 0.62 nM. The kinetic parameters for $tRNA^{Ser}$, and $tRNA^{\Delta Ser}$ were determined using RNA and SerRS concentrations identical to the conditions described elsewhere (Sampson & Saks, 1993). The individual kinetic parameters for these substrates were calculated from Eadie-Hofstee plots using the program ENZYME KINETICS (dogStar software/Indiana Univ.). Individual kinetic parameters could not be obtained for the aminoacylation of LeuΔCUA, STL8, and STL6 by LeuRS, and for the aminoacylation of STL5, STL7 and STL8 by SerRS. $V_0/[S]$ was assumed to reflect K_{cat}/K_m since the measured initial rates of aminoacylation were essentially proportional to RNA concentration indicating that the RNA concentrations were below K_m and the reaction was first order. Initial rates for all substrates were calculated from a linear least square regression using five time points whose correlation coefficients were all 0.98 or greater.

n.d.= not determined.

	LeuRS				SerRS	
tRNA Substrate	K_M (μM)	K_{cat} (sec-1)	K_{cat}/K_M (M-1.sec-1)	Loss of Specificity	K_{cat}/K_M (M-1.sec-1)	Loss of Specificity
T7 LEU UAA	0.21	3.2	$1.5x10^7$	(1.0)	n.d.	n.d.
Native Leu UAA	0.23	2.7	$1.2x10^7$	1.3	n.d.	n.d.
LEU CUA	0.22	0.70	$3.3x10^6$	4.6	n.d.	n.d.
LEU UUU	0.26	2.6	$1.0x10^7$	1.5	n.d.	n.d.
LEUΔ CUA	n.m.	n.m.	$7.6x10^4$	200	n.d.	n.d.
STL2	1.1	2.6	$2.4x10^6$	6.3	n.m.	>8300
STL8	n.m.	n.m.	$1.3x10^5$	120	$8x10^2$	8300
STL7	0.65	1.2	$1.8x10^6$	8.4	$2x10^3$	3300
STL3	3.5	2.3	$6.4x10^5$	23	n.m.	>8300
STL5	n.m.	n.m.	n.m.	>13,000	$2.15x10^3$	3100
STL6	n.m.	n.m.	$5.1x10^3$	3000	n.m.	>8300
STL9	0.93	1.7	$1.8x10^6$	8.4	$2.9x10^3$	2300
SER	n.m.	n.m.	n.m.	>13,000	$6.6x10^6$	(1.0)
SERΔ	n.m.	n.m.	n.m.	>13,000	$1.45x10^3$	4600

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