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Structure and function of Escherichia coli valine transfer RNA in aminoacylation and ternary complex formation

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Structure and function of *Escherichia coli* valine transfer RNA in aminoacylation and ternary complex formation

by

Jack Chia-Hsiang Liu

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Biochemistry

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Iowa State University

Ames, Iowa

1997

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For the Graduate College
DEDICATION

To My Wife, Emily
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INTRODUCTION

Translation is the process of protein synthesis that produces the different classes of proteins with various biochemical properties and cellular functions. Overall, the translational process requires the coordinated participation of over 100 macromolecules, including transfer RNAs (tRNA) that carry amino acids attached to their 3'-terminal adenosine, components of the ribosome, the translational factors, etc. Two domains of a tRNA molecule, the anticodon and the acceptor stem (especially the 3'-CCA end) actively participate in the translational process (Sprinzl and Cramer, 1979). tRNAs decode genetic information through base pairing of their anticodons with the codons of a programmed messenger RNA (mRNA) on the ribosome, and they transfer amino acids into a growing polypeptide chain through the interaction of 3'-CCA of tRNA with ribosomal proteins/RNA. Thus, transfer RNA molecules play a central role in gene expression to ensure the efficiency and fidelity of protein synthesis.

The efficiency and fidelity of protein synthesis depend not only on events occurring on the ribosome, but also on the accuracy of each tRNA molecule being aminoacylated with its cognate amino acid: a reaction catalyzed by aminoacyl-tRNA synthetases. Since all tRNAs adopt similar L-shaped tertiary structures, each aminoacyl-tRNA synthetase must specifically recognize its cognate tRNA while effectively discriminating against all others. The specific and accurate aminoacylation of a tRNA by its cognate synthetase is governed by a number of nucleotides in a tRNA; these nucleotides are referred to as "identity elements". Over the years, a number of biochemical, biophysical, and genetic approaches have been developed...
and refined to study the structure-function relationships of tRNA molecules in the 
aminoacylation reaction (Saks et al., 1994; Giege et al., 1993). In the present study, 
structure-function relationships of *E. coli* tRNA_{Val} were determined by studying the steady-
state kinetics of aminoacylation of *in vitro* transcribed tRNA variants. This study also 
examines the role of the universally conserved 3′-CCA sequence of tRNA_{Val} in polypeptide 
synthesis. In particular, the role of 3′-end of tRNA_{Val} in ternary complex formation with 
elongation factor Tu was examined in depth.

**Transfer RNA: Structure and Functions**

**Structure of tRNA**

Determination of the sequence of yeast tRNA_{Ala} in 1965 marked the first such 
achievement for any RNA molecule (Holley et al., 1965). From the sequence information, it 
was inferred that tRNAs can fold into a cloverleaf secondary structure (Figure 1A). 
Currently, there are almost 2000 tRNA or tRNA gene sequences known from more than 200 
organisms and organelles (Gesteland and Atkins, 1993). Girheimer et al. (1995) have 
reviewed the general features of the primary and secondary structure of tRNA. The 
cloverleaf structure of tRNA has about half of its nucleotides base paired to form four helical 
stems: the acceptor stem, D-stem, T-stem, and anticodon stem. Five groups of unpaired 
nucleotides comprise four loops and one single stranded region; these are the D-loop, the T-
loop, the anticodon loop, the variable loop, and the 3′-NCCA end. The acceptor stem 
contains both of the 3′ and 5′ termini of the tRNA, and has the amino acid attachment site at 
the 3′-terminal A of the universally conserved 3′-CCA sequence. The anticodon base triplet,
Figure 1. General tRNA structure. (A) Cloverleaf structure with bases replaced by numbers indicating the standard 76 nucleotide positions. Invariant and semi-invariant positions are designated by nucleotide symbols or R for purine, Y for pyrimidine, H for highly modified purine. Tertiary base pairs are indicated by solid lines. (B) L-shaped structure of yeast tRNA$^{\text{Phe}}$. The structure was generated by the program RasMol (R. Sayle) based on the yeast tRNA$^{\text{Phe}}$ structure (Jack and Levitt, 1978); Brookhaven Databank access code 4tna.
located in the anticodon loop, possesses the necessary information to recognize the codon on mRNA. There are 15 invariant positions that always contain the same base, and eight semi-invariant positions that always have either a purine or a pyrimidine (Figure 1A). The chain length of the known tRNAs varies from 72 to 95 nucleotides. Transfer RNAs of different length are able to conform to the cloverleaf structure because the additional nucleotides are accommodated in the variable loop and variable regions in the D-loop. The number of nucleotides in each stem and loop is generally constant except for the variable loop and the D-loop. Class I tRNAs contain 4 to 5 nucleotides in the variable loop, while class II tRNAs can have up to 21 nucleotides in this loop. The α and β regions in the D-loop (Figure 1A) vary in length from 1 to 3 nucleotides in different tRNAs.

An interesting characteristic of tRNAs is their high content of modified nucleosides which display a wonderful array of structural variations (Bjork, 1995). The degree of modification varies with the source of the tRNAs. In human tRNAs, up to one fourth of the bases may be modified whereas in bacterial tRNAs there is much less nucleoside modification. An imino proton NMR study showed that nucleoside modifications of E. coli tRNA^Val stabilize the binding of magnesium ions in tRNA^Val (Yue et al., 1994). The role of modified nucleosides is generally unclear, although a number of them have been implicated in processes ranging from restriction and expansion of codon recognition, to specific recognition by aminoacyl-tRNA synthetases. An example is the nucleoside modification of cytidine, C, to lysidine (2-lysylcytidine), L, at the first position of the anticodon of E. coli tRNA^His (Muramatsu et al., 1988). By this single modification, the codon specificity is switched from the methionine codon, AUG, to an isoleucine codon, AUA, and at the same
time, the aminoacylation identity of the tRNA is switched from methionine to isoleucine (Muramatsu et al., 1988).

The first high resolution X-ray crystal structure, that of yeast tRNA$^{\text{Phe}}$ at 3 Å, was solved in 1974 (Figure 1B) (Kim et al., 1974; Robertus et al., 1974). Kim (1976) has reviewed the general features of the three-dimensional structure of yeast tRNA$^{\text{Phe}}$. From X-ray crystallographic studies, the backbone structure of yeast tRNA$^{\text{Phe}}$ was found to have several characteristics: (1) the molecule has the overall shape of the letter “L”; (2) helical stems implied by the cloverleaf secondary structure are maintained in the three-dimensional structure; (3) the acceptor and T stems form a continuous double helix, the D and anticodon stems form another continuous double helix, and the two continuous double helices are approximately perpendicular to each other; (4) the 3'-CCA and anticodon are located at the two ends of the “L”; (5) the D and T loops come near each other to form the corner of the “L”. The overall dimension of a tRNA molecule places the 3'-CCA and the anticodon loop of tRNA approximately 75 Å apart.

There are a total of nine tertiary interactions, involving 21 nucleotides, that contribute to the folded L-shaped structure of yeast tRNA$^{\text{Phe}}$ (Figure 1B). Four of the tertiary interactions are formed by conserved nucleotides: U8-A14, G18-U55, G19-C56, and U54-A58 (Figure 1A) (Grosjean et al., 1982). The remaining five tertiary interactions are formed by semiconserved nucleotides at positions 15-58, 13-22-46, 9-23-12, 45-10-25, and 26-44 (Figure 1A). In *E. coli* tRNA$^{\text{Val}}$, the nucleotides involved in tertiary interaction are the same as the ones found in yeast tRNA$^{\text{Phe}}$, except for positions 26-44 where *E. coli* tRNA$^{\text{Val}}$ has A26-G44 instead of the G26-A44 found in yeast tRNA$^{\text{Phe}}$. From the structure of yeast
tRNA\textsuperscript{Phe}, it is shown that almost all the bases are stacked. It is believed that these extensive stacking interactions are the primary source of the stabilizing force maintaining the three dimensional structure of the tRNA, and this extensive stacking is expected to be a general feature for all tRNAs (Kim, 1976).

**Functions of tRNA**

Many biological properties of tRNAs are well defined. From its biosynthesis to its functions on the ribosome, tRNA molecules interact with many different proteins, including aminoacyl-tRNA synthetase and elongation factors (Söll and RajBhandary, 1995).

Translation of the nucleotide sequence of mRNA into the amino acid sequence of proteins, an important process in the dogma of molecular biology, is mediated by tRNAs, ribosomes and factors. The role of tRNA in protein synthesis has been reviewed by Pongs (1978) and Noller (1991). During the translational process, there are three important phases: initiation, elongation, and termination. In *E. coli*, the 70S ribosome consists of 30S and 50S subunits.

Before participating in the translational process, a tRNA molecule is aminoacylated with a specific amino acid by its cognate aminoacyl-tRNA synthetase, forming an aminoacyl-tRNA. The aminoacylation reaction, carried out in two steps, is important in the overall protein biosynthetic pathway.

\[
\text{Amino Acid} + \text{ATP} \leftrightarrow \text{Aminoacyl-adenylate} + \text{PP},
\]

\[
\text{Aminoacyl-adenylate} + \text{tRNA} \leftrightarrow \text{Aminoacyl-tRNA} + \text{AMP}
\]
In the first step of the reaction, the synthetase catalyzes the activation of its cognate amino acid, using a molecule of ATP to form an aminoacyl adenylate. In the second step, the amino acid is transferred from the aminoacyl adenylate to a hydroxyl group on the 3'-terminal nucleotide of the cognate tRNA.

In the initiation phase, the initiator tRNA, formylmethionyl-tRNA^fmet, forms a 30S initiation complex involving the 30S ribosomal subunit and the mRNA. This 30S initiation complex is joined by the 50S ribosomal subunit to form a 70S initiation complex. Formation of the initiation complexes is facilitated by the initiation factors (IF-1, IF-2 and IF-3) and GTP. In the elongation phase, aminoacyl-tRNAs form a ternary complex with elongation factor Tu (EF-Tu) and GTP; formation of the ternary complex prevents the spontaneous hydrolysis of the unstable ester linkage in aminoacyl-tRNA. The ternary complex binds to the A-site of the ribosome, directed by proper recognition between the anticodon and codon. This codon-anticodon recognition process determines the identity of an amino acid incorporated at each position of the polypeptide chain. Upon binding of the aminoacyl-tRNA to the ribosomal A-site, GTP is hydrolyzed to GDP and EF-Tu is released from the ribosome in the form of the binary complex, EF-Tu:GDP. This binary complex has a low affinity for both the ribosome and aminoacyl-tRNA. Following binding of aminoacyl-tRNA at the ribosomal A site, the amino group of the amino acid initiates a nucleophilic attack on the carboxyl group of peptidyl-tRNA in the P-site, resulting in formation of a peptide bond. This reaction occurs at the peptidyltransferase center of the 50S ribosomal subunit and may be catalyzed by ribosomal RNA (Noller, et al., 1992; Samaha, et al., 1995). The peptidyl-tRNA is then translocated from the A-site to the P-site, leaving an empty A-site ready for the next
round of the elongation cycle. This translocation from A-site to P-site is catalyzed by elongation factor G (EF-G) and also requires GTP. Uncharged tRNA leaves the P-site and moves to the exit site (E-site) before dissociating from the ribosome. Translation is terminated by release factors that recognize the termination codons and cause the hydrolysis of the ester bond of peptidyl-tRNA.

Besides the very important role of tRNA in the translational process, tRNA molecules also participate in a variety of other functions in cellular metabolism such as cell wall biosynthesis, chlorophyll and heme biosynthesis, and as primers for retroviral RNA-directed DNA synthesis, including that of HIV. These functions have recently been reviewed by Söll (1993). This versatility in the function of this small RNA molecule is one of the fascinating aspects of tRNAs.

**Role of 3′-CCA Sequence of tRNA in Aminoacylation Reaction**

The 3′-CCA sequence of tRNA is conserved in all tRNA species from prokaryotic to eukaryotic species. A number of previous studies on the structure of the 3′-CCA sequence of tRNA involved biochemical and biophysical techniques including chemical modifications, x-ray diffraction, and NMR. These results have shown that the 3′-CCA end of tRNA has an ordered structure that is stabilized by vertical stacking of the bases onto the acceptor stem (Sprinzl and Cramer, 1979). An ordered structure of the 3′-CCA sequence may be important for determining the spatial arrangement of the 3′-adenosine to facilitate the interaction of the 3′-CCA with other macromolecules, such as aminoacyl-tRNA synthetases, elongation factors, and components of the ribosome. Previous studies, using chemical and enzymatic
modifications, have found that the 3'-CCA sequence is important for aminoacylation of a
tRNA by its cognate synthetase (Sprinzl and Cramer, 1979). Modifications of either the
adenine base (Best and Novelli, 1971; Rether et al., 1974) or ribose moiety (Cramer et al.,
1968; Uziel and Jacobson, 1974) of the 3'-terminal nucleotide of tRNA resulted in a
complete loss or a dramatic decrease in aminoacylation activity of tRNA. Modifications of
the cytidines at positions 74 and 75 have different effect on the aminoacylation activities of
the modified tRNAs depending on the type of modifications. Yeast tRNA^Tyr having the 3'-
UUA sequence was shown to be actively aminoacylated (Kucan et al., 1973). Introduction
of a bulky substitution such as an alkyl group into position 75 of tRNA^Phe inhibited the
activity of this modified tRNA (Sprinzl et al., 1978). In many cases, the $K_m$ values of
modified tRNAs remain unchanged, while the $V_{max}$ values are appreciably lowered.

Effects of systematic mutations of all three bases of the 3'-CCA sequence in tRNA^Val
have been investigated in the aminoacylation of tRNA^Val by valyl-tRNA synthetase (Liu and
Horowitz, 1994). Most in vitro transcripts of tRNA^Val with altered 3'-termini are readily
aminoacylated. The presence of guanosine at position 75 or 76 severely impaired the
aminoacylation activities of these two mutants, and tRNA^Val with a 3'-UUA sequence is also
a very poor substrate for ValRS. Tamura et al. (1994) had also performed a systematic study
on the role of 3'-CCA sequence of tRNA^Val in aminoacylation and found similar results.
Moor et al. (1994) had found that E. coli and T. thermophilus tRNA^Phe with 3'-UUA and 3'-
CCC sequences are substrates for their respective synthetases; tRNA^Phe with 3'-AAA was a
poor substrate, and tRNA\textsuperscript{Phe} with 3'-UUU sequence was inactive in aminoacylation by either enzyme.

Previous studies showed that the 3'-CCA sequence of tRNA is important for the aminoacylation activity of the molecule. These studies showed that the same modifications introduced into the 3'-CCA sequence of different tRNAs have different effects on the aminoacylation activity of tRNAs, suggesting that the modes of interaction between the 3'-CCA sequence of different tRNAs and their cognate synthetases differ.

**Aminoacyl-tRNA Synthetases**

There are 20 aminoacyl-tRNA synthetases in *E. coli*, and each enzyme is responsible for the specific aminoacylation of one or more isoaccepting tRNAs with a cognate amino acid. Although the aminoacyl-tRNA synthetases catalyze the same reaction and use ATP as a common substrate, their molecular weights and structural assemblies are very diverse. Arnez and Moras (1997) have recently summarized the characteristics of the aminoacyl-tRNA synthetases. These enzymes exhibit different types of quaternary structures: \( \alpha, \alpha_2, \alpha_4, \) and \( \alpha_2\beta_2 \). The size of the polypeptide chain ranges from 334 amino acids for a subunit of homodimeric (\( \alpha_2 \)) tryptophanyl-tRNA synthetase (Hall *et al.*, 1982) to 1112 amino acids in phenylalanyl-tRNA synthetase (Mechulam *et al.*, 1984). Sequence comparison in addition to structural analysis of a limited number of three-dimensional structures allowed the classification of the synthetases into two major classes of ten synthetases each (Eriani *et al.*, 1990). Class I synthetases have two conserved sequence motifs, HIGH and KMSKS (Brick
et al., 1989; Brunie et al., 1990; Rouald et al., 1989), that are involved in ATP binding. The active site of class I synthetases has the classic dinucleotide (or Rossmann) fold consisted of a six-stranded parallel β sheet (Rossmann et al., 1976). The ten synthetases in class II do not have the two class I sequence motifs. Instead, they share other conserved sequence motifs called motif 1, 2, and 3 (Cusack et al., 1990; Ruff et al., 1991). Both motifs 2 and 3 are part of the active site that features a seven-stranded antiparallel β sheet surrounded by α helices. This classification of the synthetases based on sequence and structural information also has a functional significance. All class I synthetases attach amino acids to the 2′-OH group of the terminal adenosine of the tRNA, whereas all but one class II synthetases attach amino acids to the 3′-OH group. Phenylalanyl-tRNA synthetase, a class II synthetase that attaches phenylalanine to the 2′-OH, is the only exception (Moras, 1992).

In addition to the major classification of aminoacyl-tRNA synthetases, class I or class II synthetases can be further divided into subgroups based on additional common structural features (Delarue and Moras, 1993). GlnRS, GluRS and ArgRS are in a subgroup of class I synthetases, and this subgrouping also corresponds to the fact that they are all specific for charged amino acids and require a bound tRNA for amino acid activation (Ravel et al., 1965). Another subgroup comprises synthetases for aromatic amino acids. TyrRS and TrpRS. ValRS is in a subgroup with four other synthetases: IleRS, LeuRS, CysRS and MetRS. For the class II synthetases, AspRS, AsnRS, and LysRS are in a subgroup that contains consensus sequences at the amino terminus. SerRS, ProRS, ThrRS, and HisRS are in a subgroup that shares homologies at their carboxy termini. Finally, GlyRS, PheRS, and AlaRS are grouped into a subgroup that shows distinctive quaternary features.
Recognition of tRNAs by Aminoacyl-tRNA Synthetases

Methodology For Studying Recognition of tRNA by Aminoacyl-tRNA Synthetase

TRNAs are well-characterized RNA molecules of known three dimensional structure based on the crystal structure of yeast tRNA^Phe. Because of the structural similarity of the tRNAs, and the chemical similarity of aminoacylation systems, the discrimination of tRNA molecules by aminoacyl-tRNA synthetases (aaRS) remains a challenging and intriguing problem in molecular recognition (McClain, 1993; Giegé et al., 1993).

There are two general approaches utilized by researchers in this field to study the identity of tRNAs. Both methods involved the genetic engineering of tRNA genes in vitro to produce mutants at specific positions in a tRNA molecule. Recognition of tRNA by synthetase is then determined by either examining the function of mutant amber suppressor tRNAs in vivo, or by studying the aminoacylation kinetics of in vitro transcripts of mutant tRNAs. In vivo assays of amber suppressor tRNA activity use a mutant dihydrofolate reductase gene as a reporter protein. In this mutant dihydrofolate reductase mRNA, the codon for the amino acid at position 10, near the amino terminus, is replaced by an amber termination codon (CUA) (Normanly et al., 1986). The amino acid specificity of mutant suppressor tRNAs is then determined by identifying the amino acid inserted at position 10 (amber site) of dihydrofolate reductase by amino acid sequence analysis. Schulman (1991) has reviewed identity elements of several tRNAs obtained by the in vivo approach.

The inability to test tRNAs containing wild type anticodons is a major drawback of the in vivo amber suppressor assay. A major advance in the in vitro synthesis of RNA was the use of phage promoters and phage RNA polymerases (Melton et al., 1984). Currently,
the method of choice, first employed by Sampson and Uhlenbeck (1988) in the study of yeast tRNA\textsuperscript{\textsc{pec}} for \textit{in vitro} tRNA recognition studies, uses the T7 promoter and T7 RNA polymerase for the run-off transcription of a cloned tRNA gene. The aminoacylation activity of many of the \textit{in vitro} transcribed tRNAs with wild type sequences is very similar to that of their native counterparts, even though they lack all of the naturally occurring nucleoside modifications. This is true for \textit{E. coli} tRNA\textsuperscript{Val} (Chu and Horowitz, 1988) which is used in the present study. Typically, \textit{in vitro} recognition studies are carried out by measuring the rate of aminoacylation of tRNA variants with base substitutions at positions under investigation. The relative efficiency of a tRNA as a substrate for an aminoacyl-tRNA synthetase is evaluated by comparing \(k_{\text{cat}}/K_m\) or \(V_{\text{max}}/K_m\), the "specificity constant."

**Synthetase Recognition Elements of tRNA**

A long-standing question in the study of tRNA identity has been: What is the molecular basis by which an aminoacyl-tRNA synthetase recognizes its cognate tRNAs with such a high degree of precision, and discrimines against other tRNAs with similar overall secondary and tertiary structures? Specificity of the genetic code depends absolutely on the attachment of a correct amino acid to a tRNA by the corresponding aminoacyl-tRNA synthetase (aaRS) (Schimmel, 1989; Normanly and Abelson, 1989). Despite the relatively large number of nucleotides in tRNAs, experimental results indicate that only a limited number of nucleotides contribute to the specific recognition of tRNAs by cognate synthetases. Many studies have been carried out, in \textit{E. coli} at least, which allow a general pattern of tRNA recognition to emerge. In most tRNAs, the anticodon, the discriminator base, and parts of the acceptor stem sequences are implicated in the recognition process.
(Pallanck et al., 1995; Saks et al., 1994). Each tRNA is believed to contain within its sequence a set of 'recognition nucleotides' that allows productive recognition and aminoacylation by the cognate synthetase and perhaps another set of 'negative determinants' that blocks the aminoacylation of non-cognate tRNAs. Together these define the 'identity elements' of a tRNA. A great deal of progress has been made in locating the nucleotides of particular tRNA molecules that specify aminoacylation identity.

**Anticodon recognition**

Since the anticodon positions 34, 35, and 36 specify the decoding capacity of a tRNA, it would seem to be a natural place to specify tRNA acceptor identity as well (Fralova and Kisselev, 1964). The role of the anticodon in the aminoacylation of tRNA has recently been reviewed by Pallanck et al. (1995). Significant reductions in aminoacylation activities following nucleotide replacements in the anticodon have been observed for 15 of 20 *E. coli* tRNAs; those for Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Met, Phe, Pro, Thr, Trp, Tyr, and Val (amino acids designated by their three-letter codes). Two additional tRNAs, Glu and Lys, also have reduced activity upon base substitutions in the anticodon, but to a lesser degree. In the case of tRNA^{Ala} and tRNA^{Ser}, the cognate synthetase does not interact with the anticodon loop. For some tRNAs, such as tRNA^{Ile}, tRNA^{Met}, and tRNA^{Phe}, nucleotides at all three positions of the anticodon are important for recognition. In others, such as tRNA^{Thr}, tRNA^{Gly}, and tRNA^{Val}, only two nucleotides of the anticodon are important for recognition. The effect of base substitutions in the anticodon varies from one tRNA to the next, possibly reflecting the relative importance of the anticodon in recognition of different tRNAs. While in most cases base modification of nucleotides in the anticodon is not essential for the
recognition of tRNAs by their cognate synthetases, in the case of *E. coli* tRNA^Val^ it is the modified base lysidine, a derivative of cytidine, that is recognized by isoleucyl tRNA synthetase (Muramatsu *et al.*, 1988).

Although a decrease in aminoacylation on nucleotide substitution may indicate that a recognition element has been eliminated, other interpretations are possible, e.g., effects on the tertiary structure of tRNA. The most convincing way to demonstrate that an anticodon dictates the aminoacylation of a tRNA is to replace the anticodon of one tRNA with that of another and show that the amino acid acceptor identity of the tRNA coincides with the anticodon. Schulman and Pelka (1988) performed a series of elegant experiments that constitute "identity swap experiments" to show the contribution of the anticodon to the identity of *E. coli* tRNA^Val^ (UAC) and tRNA^Met^ (CAU). Sampson *et al.* (1989) have successfully demonstrated, with identity swap experiments, that five nucleotides are all that are required for the specific recognition of yeast tRNA^Phe^ by phenylalanyl-tRNA synthetase (PheRS), provided that these recognition nucleotides are positioned correctly in the secondary and tertiary structures of the tRNA.

How do nucleotides in the anticodons contribute to the recognition of tRNAs? Biochemical studies of tRNA-synthetase complexes suggest conformational changes in the anticodon of tRNAs during aminoacylation (Frugier *et al.*, 1992). Crystallographic studies of the *E. coli* tRNA^Gln^ -GlnRS (Rould *et al.*, 1991) and the yeast tRNA^asp^ -AspRS (Ruff *et al.*, 1991) complexes show significant conformational changes in the anticodon loop of the tRNAs as a result of tRNA-synthetase interaction. Since the anticodon is the important identity site in many tRNAs, it is believed that the effects of recognition of anticodon
determinants must be transmitted to the synthetase's catalytic site, about 75 Å away from the anticodon of the tRNA, by conformational changes of the synthetase, the tRNA or both. Ivana et al. (1994) identified, by genetic selection, a 17 amino acid loop that is required for transferring the information of anticodon recognition to the active site of glutaminyl-tRNA synthetase (GlnRS). Rogers et al. (1994) also found amino acid residues interacting with the inside of the L-shaped tRNA to be responsible for the role of transmitting anticodon recognition to the active site of GlnRS.

**Acceptor stem and discriminator base recognition**

The acceptor stem and the discriminator base (position 73) of a tRNA, because of their close proximity to the catalytic site of aminoacyl-tRNA synthetases, would also be logical locations for identity elements. For 17 out of 20 *E. coli* tRNAs, all except tRNA*^Phe^*, tRNA*^Thr^*, and tRNA*^Ser^* the discriminator base serves as a recognition nucleotide (reviewed by Pallanck et al., 1995). Base pairs in the acceptor stem also have been found to contain important recognition elements for many tRNAs, including *E. coli* tRNA*^Ala^*, tRNA*^Gin^*, tRNA*^Gly^*, tRNA*^His^*, and tRNA*^Ser^*. For tRNA*^Ala^*, a G:U base pair in the 3′:70 positions of the acceptor stem is the most important identity element (Hou and Schimmel, 1988; McClain and Foss, 1988). In fact, a RNA minihelix (Francklyn and Schimmel, 1989), a 12-base pair stem and loop RNA molecule that consisted of sequences from the acceptor stem and the T-stem and loop of tRNA*^Ala^* plus the 3′-ACCA terminus, as well as a small RNA molecule with a four-base pair stem (same sequence as the first four base pairs in the acceptor stem of tRNA*^Ala^*) connected by a tetraloop and the 3′-ACCA terminus (Shi et al., 1992) are substrates for alanyl-tRNA synthetase provided that the G3:U70 base pair is present. The
minihelix shows only a five-fold reduction in aminoacylation activity compared to full-length tRNA^{Ala}. Other studies showed that the first three base pairs in the acceptor stem of tRNA^{Ser} are important identity elements of this tRNA (Normanly et al., 1986). tRNA^{His} is unique among E. coli tRNAs in having an extra base pair in the acceptor stem (-1:73), and as a result, this tRNA contains 8 base pairs in its acceptor stem. The -1G:C73 base pair is important for the aminoacylation of this tRNA (Himeno et al., 1989).

Variable pocket and variable loop recognition

In the crystal structure of yeast tRNA^{Phe} (Kim et al., 1974; Robertus et al., 1974), nucleotides 16, 17, 20, 59 and 60 reside in close proximity to one another and form a so-called variable pocket. In addition to the anticodon loop, the acceptor stem and the discriminator base, recognition nucleotides in the variable pocket and the variable loop have been identified in a few tRNAs. In E. coli tRNA^{Phe}, besides the major recognition nucleotides at all three positions of the anticodon, positions 20, 59 and 60 in the variable pocket and positions 44 and 45 in the variable loop all contribute modestly to recognition (Peterson and Uhlenbeck, 1992). Furthermore, position 20 of yeast tRNA^{Phe} was found to be involved in recognition by yeast PheRS, in addition to position 73 and the major recognition nucleotides in the anticodon (Sampson et al., 1989). In E. coli tRNA^{Arg}, position 35 is the major identity element for synthetase recognition, however A20, a nucleotide found exclusively in E. coli tRNA^{Arg} isoacceptors (McClain et al., 1990), was shown to be important for recognition, by in vivo studies (McClain and Foss, 1988; Normanly et al., 1990).
Importance of structural features in tRNA recognition

Although tRNAs have similar global structure as dictated by the common protein synthesis machinery, there are subtle conformational differences among tRNAs. Giege et al. (1993) have summarized some examples of conformational features of a tRNA that influence aminoacylation efficiency. It has been found that transplantation of foreign tertiary interactions into the sequence context of yeast tRNA\(^{\text{Phe}}\) has little effect on the steady-state aminoacylation kinetics catalyzed by PheRS (Sampson et al., 1990). However, transplantations of tertiary interactions found in yeast tRNA\(^{\text{Phe}}\) into yeast tRNA\(^{\text{A} \text{sp}}\) alter the conformation of the tRNA, based on lead mapping studies (Perret et al., 1992). Studies on identity swap experiments with yeast tRNA\(^{\text{A} \text{sp}}\) and tRNA\(^{\text{Phe}}\) reached some general conclusions: (1) transplantation of the identity set may not be sufficient to confer optimal new specificity, (2) synthetases have variable sensitivities to the conformation of their tRNA substrates, (3) manipulation of tRNA conformation can overcome some of the constraints that attenuate the efficiency of the specificity switch. More recently, Senger and Fasiolo (1996) also found that tRNA tertiary structure makes an important contribution to the identity of yeast tRNA\(^{\text{Met}}\). The importance of tertiary interactions and the conformation of tRNA in the aminoacylation reaction has begun to attract more attention in the studies of synthetase recognition nucleotides in tRNAs.

**Synthetase Recognition Elements of E. coli tRNA\(^{\text{Val}}\)**

The role of the anticodon of tRNA\(^{\text{Val}}\) (Figure 2) in ValRS recognition has been very well established (Schulman and Pelka, 1988; Tamura et al., 1991; Chu et al., 1992). A35 and C36 are the major synthetase recognition determinants of tRNA\(^{\text{Val}}\). McClain and Nicholas
Figure 2. Cloverleaf structure of *E. coli* tRNA\(^{Val}\) (shown without modified bases).
(1987) used computer analysis to identify nucleotide positions that offer distinguishing characteristics of each tRNA acceptor type. Based on the available *E. coli* and *Salmonella* tRNA sequences, their analysis suggested that possible recognition nucleotides for tRNA\(^{\text{Val}}\) are A35 and C36 in the anticodon loop, the U4:A69 base pair in the acceptor stem, the C30:G40 base pair in the anticodon stem, G44 in the variable loop, and the C51:G63 base pair in the T-stem. Schulman and Pelka (1988) suggested that additional nucleotides outside of the anticodon may make some contribution to tRNA\(^{\text{Val}}\) identity, since tRNA\(^{\text{Met}}\) with a tRNA\(^{\text{Val}}\) anticodon, UAC, is only one tenth as efficient a substrate for ValRS as wild type tRNA\(^{\text{Val}}\)(UAC). The acceptor stem of tRNA\(^{\text{Val}}\) makes contact with ValRS during the aminoacylation reaction, as suggested by an \(^{19}\text{F}\) NMR study of ValRS interaction with 5-fluorouracil (FU) modified tRNA\(^{\text{Val}}\) (Chu and Horowitz, 1991) and by nuclease footprinting studies on tRNA\(^{\text{Val}}\) (Derrick, 1991). More recently, Liu (1995) has identified A73 and G20 as additional recognition nucleotides of tRNA\(^{\text{Val}}\). Experimental results in our laboratory indicate that the eight modified bases in native *E. coli* tRNA\(^{\text{Val}}\) do not play a significant role in the recognition process (Chu and Horowitz, 1989). Furthermore, incorporation of the uracil analog, 5-fluorouracil (FU), into tRNA\(^{\text{Val}}\) and the complete assignment of the \(^{19}\text{F}\) NMR spectrum of 5-fluorouracil-substituted tRNA\(^{\text{Val}}\) make \(^{19}\text{F}\) NMR a powerful tool for probing the solution structure of FU-substituted tRNA\(^{\text{Val}}\) (Chu and Horowitz, 1991; Chu *et al.*, 1992).
Recognition of tRNAs by Elongation Factor Tu

Biochemical Studies of Aminoacyl-tRNA and Elongation Factor Tu Interaction

During protein synthesis in bacteria, elongation factor Tu recognizes, transports, and positions the codon-specified aminoacyl-tRNA onto the A site of the ribosome. In order for the elongation process of translation to occur, elongation factor Tu has to recognize all aminoacylated elongator tRNA molecules in the cell (Faulhammer and Joshi, 1987). Ternary complex formation of aminoacylated elongator tRNA with elongation factor Tu is a crucial step for cell viability since formation of this complex permits the controlled entrance of aminoacyl-tRNA into the elongation cycle (Clark et al., 1990).

Elongation factor Tu (EF-Tu) of *E. coli* is a monomeric protein with a molecular mass of 43,200 daltons, containing 393 amino acid residues. The structure of EF-Tu in the GDP form (Clark et al., 1990) consists of three domains, in a triangular arrangement. Domain I has residues 1-200 and is roughly spherically shaped. It is the site of the GTPase activity of the molecule and is therefore called the G domain (Bourne et al., 1991). Domain II (residues 209-299) and domain III (residues 300-393) are barrel-shaped. EF-Tu is the most abundant protein in the *E. coli* cell, comprising about 5% of the total cellular proteins. The high level of EF-Tu is needed to sequester aminoacyl-tRNAs and prevent wasteful hydrolysis of the aminoacyl linkage to tRNA. This excess of EF-Tu over aminoacyl-tRNA also increases the accuracy of translation (Pingoud et al., 1990).

Since all tRNA sequences differ from each other, except for a set of conserved and semiconserved residues involved in maintaining the classical L-shaped folding, elongation factor Tu has to recognize some characteristic structural features or conserved sequences
carried by all elongator tRNA species. The process of recognition between tRNAs and translation factors is understood only in some limited cases.

Nazarenko et al. (1994) reported that conserved and semiconserved nucleotides do not contribute significantly to the interaction of tRNA\(^{\text{Phe}}\) transcripts with EF-Tu. Faulhammer and Joshi (1987) have reviewed the structural features of aminoacyl-tRNAs that are required for recognition by elongation factor Tu. Several regions of elongator tRNAs have been implicated as important sites for the interaction with EF-Tu:GTP including the aminoacyl moiety, the 3'-CCA end, and the continuous helix of the acceptor arm domain. In the EF-Tu:aminoacyl-tRNA complex, the T loop and the anticodon are free and accessible for interaction with the ribosomal A site and the messenger RNA, respectively. Enzymatic probing shows that parts of the amino acid stem, the T stem, and most of the variable loop of tRNAs in the ternary complex are protected from nuclease digestion by EF-Tu (Wikman et al., 1982). Cross-linking experiments suggest that parts of the T loop and stem interact directly with EF-Tu (Wikman et al., 1987). Furthermore, Joshi et al. (1984) demonstrated, using a valylated tRNA-like domain derived from turnip yellow mosaic virus RNA, that a RNA fragment containing the equivalent of the variable loop and the amino acid acceptor stem of tRNA is able to bind with \textit{E. coli} elongation factorTu and GTP. These results agree well with a report that aminoacylated tRNAs with the anticodon stem and loop substituted by a tetranucleotide, bind to EF-Tu as well as the corresponding full-length tRNAs (Nazarenko and Uhlenbeck, 1995). Taken together, most biochemical and biophysical studies show strong evidence that the T-stem, the aminoacyl stem, the 3'-NCCA end, and the variable loop of tRNA provide the major contact points with \textit{E. coli} elongation factor Tu. Despite these
investigations, the molecular recognition between elongator aminoacyl-tRNAs and EF-Tu:GTP is just beginning to be understood, mainly through the recently solved crystal structure of a ternary complex (Nissen et al., 1995).

Non-elongator aminoacyl-tRNAs do not form a ternary complex with EF-Tu:GTP. In prokaryotes, an unusual C1:A72 mismatch at the end of acceptor stem of initiator tRNA, tRNA\textsuperscript{Met}, is responsible for the weak interaction of this tRNA with EF-Tu:GTP (RajBhandary and Chow, 1995). Mutants of tRNA\textsuperscript{Met} with a Watson-Crick base pair at base pair 1:72 interact with EF-Tu and can act as elongator tRNAs in protein synthesis (Seong and RajBhandary, 1987; Seong et al., 1989). Initiator tRNA\textsuperscript{Met} from plants and fungi are discriminated against by eukaryotic elongation factor 1\alpha (EF-1\alpha) because they have a bulky 2'-phosphoribosyl modification at purine 64 in the T-stem (Kiesewetter et al., 1990). In the discrimination against selenocysteine-specific tRNA\textsuperscript{Sec} by EF-Tu, the extra length of the acceptor stem and T-stem, 13 base pairs instead of the 12 found in other elongator tRNAs, was reported to be the critical factor in preventing EF-Tu binding (Baron and Bock, 1991).

More recently, Rudinger et al. (1996) have identified three base pairs in the acceptor arm of tRNA\textsuperscript{Sec} as the antideterminants that prevent recognition of tRNA\textsuperscript{Sec} by EF-Tu.

**Crystallographic Study of a Ternary Complex**

The crystal structure of the ternary complex of yeast phenylalanyl-transfer RNA (phenylalanyl-tRNA\textsuperscript{Phe}), elongation factor Tu (EF-Tu) and the guanosine triphosphate (GTP) analog GDPNP at 2.7 Å (Figure 3) shows many details of the recognition of aminoacyl-tRNA by EF-Tu (Nissen et al., 1995). The ternary complex was found to be a trimer, but the authors believe that this is the result of crystal packing and is not of biological significance.
Figure 3. Structure of the ternary complex (EF-Tu·Phe-tRNA\textsuperscript{Phe}·GTP analogue). EF-Tu is depicted with spacefilled model in gray, and Phe-tRNA is shown with backbone in black. The 3'-CCA-Phe is emphasized with stick model and enlarged in the inset. The three domains of EF-Tu are labeled, I, II and III, respectively. The structure was generated by the program RasMol (R. Sayle) based on the crystal structure of ternary complex (Nissen \textit{et al.}, 1995); Brookhaven Databank access code 1ttt.
The shape of a single ternary complex resembles a corkscrew. All three domains of the elongation factor Tu are involved in the binding of phenylalanyl-tRNA\textsuperscript{Phe}. However, only small areas of both the protein and the RNA make significant contact. In the crystallographic structure of the ternary complex, there are three major regions of contact involved in the binding of phenylalanyl-tRNA to EF-Tu:GDPNP: (1) the 3'-CCA-Phe binds to the domain I and II interface, (2) the 5' end and the acceptor stem of the tRNA bind at the intersection of the three protein domain interfaces, and (3) the T-stem binds to the surface of domain III. The observation that a RNA minihelix having a minimum of 10 base pairs in the helical stem is the smallest such structure able to form a tight complex with EF-Tu:GTP (Rudinger \textit{et al.}, 1994) agrees well with the observation in the crystal structure that the EF-Tu binding site on aminoacyl-tRNA covers the first 10 base pairs of the acceptor helix. Finally, the 3'-terminal adenosine of yeast tRNA\textsuperscript{Phe}, identified from its electron density in the crystal structure, is located in a hydrophobic pocket. In this pocket, the conserved residue Glu271 stacks on one side of the adenine and the conserved residues Val237 and Ile231, together with Leu289 make a hydrophobic platform for the adenine on the other side. However, EF-Tu does not make any base-specific contact (i.e. hydrogen bond) with the functional groups on the 3'-adenine.

\textbf{Role of the 3'-CCA Sequence of tRNA in Ternary Complex Formation}

The 3'-CCA sequence of tRNA is universally conserved in all tRNAs from prokaryotic and eukaryotic systems. The 2'-OH or 3'-OH of the 3'-terminal adenosine is the site of amino acid attachment. The presence of an aminoacyl moiety on the tRNA is essential for the formation of a ternary complex between an elongator tRNA and EF-Tu:GTP.
(Gordon, 1967; Lockwood et al., 1971). However, uncharged tRNA or modified tRNAs lacking all or part of the 3'-CCA end were found to influence the GTPase activity of EF-Tu (Picone and Parmeggiani, 1983).

Studies of the role of 3'-CCA in ternary complex formation are hindered by the fact that modification of the 3'-CCA sequence of tRNA often results in either a total loss or a dramatic decrease in aminoacylation activity of the tRNA. Sprinzl and Cramer (1979) have reviewed the interaction of the 3'-CCA end of tRNA with the elongation factor Tu. In one study, the 3'-CCA of yeast tRNA\textsuperscript{Phe} was extended by a cytosine to 3'-CCCA (Thang et al., 1972). Its aminoacylation activity and its ability to form ternary complex with EF-Tu.GTP are severely reduced. Elongation factor Tu can form stable ternary complexes with several 3'-CCA modified aminoacyl-tRNAs. These modifications include 5-iodocytidine (i\textsuperscript{5}C) and 2-thiocytidine (s\textsuperscript{2}C). Yeast phenylalanyl-tRNA\textsuperscript{Phe} -i\textsuperscript{5}C-i\textsuperscript{5}C-A, phenylalanyl-tRNA\textsuperscript{Phe} -C-i\textsuperscript{5}C-A, and phenylalanyl-tRNA\textsuperscript{Phe} -C- s\textsuperscript{2}C-A are active in elongation factor-dependent binding to ribosomes from rabbit reticulocytes (Baksht et al., 1977). When the 3'-terminal adenosine of yeast tRNA\textsuperscript{Phe} is replaced by formycin (F) (its absorption and fluorescence properties are useful in probing enzyme-substrate interactions), yeast tRNA\textsuperscript{Phe} -C-C-F was found to participate in \textit{in vitro} protein biosynthesis using a rabbit reticulocyte cell-free system, although at a lower level of activity than that of the unmodified tRNA\textsuperscript{Phe} (Baksht and Groot, 1975). Several derivatives of pyrrolopyrimidine, a class of nucleotide analogs used as antibiotics (Acs et al., 1964), including tubercidin, toyocamycin, and sangivamycin can be incorporated into the 3'-terminal position of rabbit liver tRNAs (Uretsky et al., 1968).
molecules with either toyocamycin or sangivamycin 3'-termini were found to be very poor substrates in the aminoacylation reaction. Only tRNA molecules with tubercidin at the 3'-termini had near-normal activity in aminoacylation and polypeptide synthesis.

Recognition of aminoacyl-tRNA is severely affected by modification of the ribofuranosyl moiety on the 3'-terminal adenosine. Ofengand and Chen (1972) showed in yeast tRNA\textsubscript{Phe} that periodate cleavage of the 2',3'-carbon-carbon bond of the 3'-terminal adenosine did not significantly affect its aminoacylation by phenylalanyl-tRNA synthetase, but this ribofuranose-modified phenylalanyl-tRNA\textsubscript{Phe} was unable to form a ternary complex with EF-Tu:GTP. This loss of activity was attributed to the conformational disturbances at the 3' end of tRNA as a result of opening the ribose ring (Chinali et al., 1974).
EXPERIMENTAL PROCEDURES

Materials

**Bacterial Strains and Plasmids**

_E. coli_ TG1 (K12, [lac-pro], SupE, thi, hsdS5/F′traD36, proA-B⁻, lacI², lacZM15), used for phagemid propagation was obtained from Amersham Life Sciences. Bacteriophage M13K07 and phagemid pUC119 were gifts from Dr. Alan Myers (Iowa State University). Phagemid pVAL119 is a derivative of pUC119 which contains a T7 promoter directly followed by a tRNA⁰⁰ gene (Chu and Horowitz, 1989). Phagemid pFVAL119 is a derivative of pVAL119 which has a Fok I restriction site inserted downstream of the tRNA⁰⁰ gene (Liu and Horowitz, 1993). Phagemid pFALA119 (Liu, 1995), pFPHE119 (Liu, 1995), pFYPHE119 are derivatives of pUC119 which contain a T7 promoter followed by _E. coli_ tRNA⁰⁰, _E. coli_ tRNA⁰⁰, and yeast tRNA⁰⁰ genes, respectively, and a Fok I restriction site. Expression plasmid pQECtuf (Ribeiro et al., 1995) containing the gene for _T. thermophilus_ elongation factor Tu was kindly provided by Dr. Mathias Sprinzl (Bayreuth University).

**Restriction Endonucleases and Enzymes**

Restriction endonucleases were purchased from New England Biolabs and Promega. T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs. Inorganic pyrophosphatase was obtained from Boehringer Mannheim Biochemicals. Valyl-tRNA synthetase (ValRS) was purified by Dr. Wen-Chy Chu from _E. coli_ strain GRB238 carrying the plasmid pHOV1 (Chu and Horowitz, 1991). T7 RNA polymerase was purified
from *E. coli* BL21/pAR1219 according to the method of Zawadzki and Gross (1991). *E. coli* S-100 was purified by Dr. Mingsong Liu according to the method of Muench and Berg (1966). Purified alanyl-tRNA synthetase was a gift from Dr. Paul Schimmel (Massachusetts Institute of Technology).

**Nucleic Acids**

Native *E. coli* tRNA\(^{Val}\) was purchased from Subriden RNA. Unfractionated *E. coli* tRNA was from Plenum Scientific Research Inc. Nucleotide triphosphates and guanosine 5′-monophosphate for *in vitro* transcription were obtained from United States Biochemical Co. and Sigma. Deoxyoligonucleotides were synthesized by the DNA Synthesis Facility at Iowa State University. Automated DNA sequencing was performed by the DNA sequencing facility at Iowa State University. The DNA sequencing kit was from Amersham Life Sciences.

**Radioisotopes and Chemicals**

\(^{3}H\)valine (27-32 Ci/mmol, 1Ci/ml), \(^{3}H\)alanine (50Ci/mmol, 1Ci/ml), \(^{3}H\)phenylalanine (65Ci/mmol, 1Ci/ml), and deoxyadenosine 5′-α-[\(^{35}S\)]thiotriphosphate were purchased from Amersham Life Sciences. Most chemicals and antibiotics (ampicillin, kanamycin, and tetracyclin) were from Sigma or Fisher Chemical Co. Biosafe NA scintillation fluor was obtained from Research Product International. Whatman 3MM paper was from the Whatman Co. Ingredients for cell culture media were from Difco Chemical Co. All other chemicals were of reagent grade or higher. The site-directed mutagenesis kits were from Amersham Life Sciences and Clonetech.
Methods

Construction of Phagemid pFYPHE119

p67YF0 (Sampson and Uhlenbeck, 1988), a derivative of pSP65 which contains the yeast tRNA^{Phe} gene, was kindly provided by Dr. Olke Uhlenbeck (University of Colorado, Boulder). Directional cloning was utilized to construct phagemid pFYPHE119 (Maniatis et al., 1989). Plasmid p67YF0 was digested with BamHI and PstI. A 101-mer fragment containing the tRNA^{Phe} gene was isolated by gel electrophoresis in 1% agarose (Amresco, agarose 3:1 high resolution blend). The DNA band corresponding to the insert was cut from the gel and purified by a gel extraction kit from Qiagen Inc. A small 44-mer DNA fragment, containing a Fok I restriction site, was isolated from BamHI and Hind III double digested pFVAL119 by electrophoresis on a 2% agarose gel, and purified as described above. The vector was prepared from pVal119 by endonuclease digestion with Pst I and Hind III, and the largest DNA fragment was isolated on a 1% agarose gel, and purified as described. The 101-mer and the 44-mer were ligated into the vector with T4 DNA ligase at 16°C overnight, and used to transformed competent TG1 cells. The sequence of the recombinant phagemid containing the tRNA^{Phe} gene was confirmed by the dideoxy DNA sequencing method of Sanger (Sanger et al., 1980).

Site-Directed Mutagenesis

Site-directed mutagenesis of tRNA genes was performed either by the method of Eckstein (Taylor et al., 1985) using a kit from Amersham Life Sciences or by the method of Deng and Nickoloff (1992) using a kit from Clonetech. Mutant clones were selected by single-lane dideoxy sequencing (Sanger et al., 1980) or the results of endonuclease digestion
patterns. The sequence of tRNA mutants was confirmed by manual or automated sequencing.

**In vitro Transcription of tRNA Genes by T7 RNA Polymerase**

All recombinant phagemids used for *in vitro* transcription were purified by cesium chloride density gradient ultracentrifugation (Maniatis *et al.*, 1989) or with the plasmid preparation kit from Primm Labs, Cambridge, MA. *Bst*NI or *Fok* I digested phagemids served as template for T7 RNA polymerase. The transcription reaction was carried out in 40 mM Tris-HCl (pH 8.0), 20 mM MgCl₂, 2 mM spermidine, 5 mM DTT, 50 μg/ml BSA, 80 μg/ml *Bst*NI or *Fok* I digested phagemid, 4 mM each of ATP, CTP, GTP, and UTP, 16 mM GMP, 80 units/μl of T7 RNA polymerase, and 4 units/ml of inorganic pyrophosphatase. After incubation for 4 hours at 42°C, the reaction mixture was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), followed by extraction with chloroform/isoamyl alcohol (24:1). Transcription products were precipitated with three volumes of 95% ethanol after addition of 1/10 volume of 3 M sodium acetate (pH 4.8).

**HPLC Purification of tRNA Transcripts**

Crude tRNA transcripts were separated from the unincorporated nucleotides and DNA fragments by HPLC on a Toyopearl DEAE-650S column (250x4.6 mm). The column was equilibrated with 25 mM Tris-HCl (pH 7.4) and 250 mM NaCl, and tRNA was eluted with a 20-ml linear gradient from 350 to 600 mM NaCl in 25 mM Tris-HCl (pH 7.4). The eluted tRNA was precipitated with three volumes of 95% ethanol. tRNA transcripts were further purified by HPLC on a Vydac C4 reverse phase column (250x4.6 mm). The column was first equilibrated with 100% buffer A (1.0 M NH₄(OAc), pH 6.0, 20 mM MgCl₂). After
injection of the sample, the column was developed with a linear gradient from 100% buffer A to 50% buffer B (10 mM NH₄(OAc), pH 6.0, 20 mM MgCl₂, and 20% methanol). The eluted tRNA was precipitated with three volumes of 95% ethanol. Finally, the tRNA sample was dried in a Savant speed-vac concentrator and dissolved in TM2 buffer (Tris-acetate pH 7.4, 15 mM magnesium acetate).

**Aminoacylation Plateau Charging Assay**

Aminoacylation of purified tRNA transcripts with valine was determined at 37°C in a 60 µl reaction mixture containing 100 mM HEPES (pH 7.5), 10 mM KCl, 15 mM MgCl₂, 7 mM ATP, 1 mM DTT, 99 µM [³H] valine, 0.05 to 0.1 A₂₆₀ of purified tRNAs, and an excess amount of ValRS (0.04 µg/µl). Ten µl aliquots were taken at different incubation times up to 60 minutes and spotted onto Whatman 3 MM filter paper which had been pre-soaked with 10% trichloroacetic acid (TCA) to stop the reaction. The paper was washed 3 times with cold 5% TCA and two times with cold 95% ethanol. After being dried, radioactivity on the paper was determined by liquid scintillation counting in Biosafe NA scintillation fluor.

Plateau levels of charging tRNA⁰ Ala transcripts with alanine were determined at 37°C, in a 60 µl reaction mixture containing 150 mM potassium phosphate (pH 7.5), 10 mM MgCl₂, 20 mM DTT, 4 mM ATP, 0.1 mg/ml BSA, 4 units of pyrophosphatase, 43 µM [³H] alanine, and purified alanyl-tRNA synthetase (Park et al., 1989). Ten µl aliquots were taken at different time points and spotted on Whatman 3 MM filter paper. The filter paper was processed as described for aminoacylation with valine.
Plateau levels of charging tRNA^Phe with phenylalanine were determined at 37°C, in a 60 μl reaction mixture containing 30 mM HEPES (pH 7.5), 2 mM ATP, 25 mM KCl, 15 mM MgCl₂, 4 mM DTT and 25 μM [³H] phenylalanine, and 0.04 μg/μl partially purified phenylalanyl-tRNA synthetase (Peterson and Uhlenbeck, 1992). Ten μl aliquots were taken at different time points and spotted on Whatman 3 MM filter paper. The filter paper was processed as described for aminoacylation with valine.

**Aminoacylation Kinetics of tRNA Variants**

Aminoacylation kinetic experiments were carried out at 37°C, in a 60 μl reaction mixture containing 100 mM HEPES pH 7.5, 10 mM KCl, 15 mM MgCl₂, 7 mM ATP, 1 mM DTT, 99 μM [³H] valine, and 1 nM purified ValRS. tRNA concentrations normally ranged from 0.5 μM to 6.0 μM as determined by the plateau charging assay. Ten μl were removed at 30-second intervals, spotted on Whatman 3 MM paper, and processed as described for the aminoacylation plateau charging assay. Initial velocities were obtained at five different concentrations of tRNA. Kᵣ and Vₘₐₓ values were determined by a least-square fit of the double-reciprocal plot of the data using the Enzfitter computer program. The results reported are the average of two or more determinations which agreed within ± 15%.

**Isolation of Aminoacyl-tRNAs**

Aminoacylation of HPLC purified *E. coli* tRNA^Val, tRNA^Ala or tRNA^Phe variants was carried out at 37°C as described under aminoacylation plateau charging assay for the respective tRNAs. When the plateau charging level of aminoacylation was reached, as determined by taking small aliquot of sample for analysis, the reaction was stopped by...
addition of an equal volume of phenol/choroform/isoamyl alcohol (25:24:1). The mixture was then extracted once with chloroform/isoamyl alcohol (24:1). Aminoacylated tRNA was precipitated with ethanol, and the pellet washed with 70% ethanol, dried, and dissolved in a small volume of 5 mM potassium acetate, pH 5.4. The sample was then loaded onto a column of Sephadex G-25 (coarse grade, Pharmacia, Inc.) that had previously been equilibrated with the same buffer. Elution with the same buffer yielded aminoacyl-tRNA freed of low molecular weight contaminants including radioactive valine. The eluted aminoacyl-tRNA was recovered by ethanol precipitation, followed by washing once with 70% ethanol, and was dissolved in 5 mM potassium acetate, pH 5.4.

**Purification of Tight-Coupled *E. coli* 70S Ribosome**

This purification procedure was based on the method of Pestka and Erbe (Pestka, 1968; Erbe et al., 1969). *E. coli* BL21 (DE3) was grown at 37°C to mid-log phase and collected by centrifugation. From this point on, all steps in the procedure were performed at 4°C. The collected cells were washed with buffer A: 20 mM HEPES (pH 7.5), 10.5 mM Mg(OAc)$_2$, 0.5 mM EDTA, 60 mM NH$_4$Cl, and 10 mM β-mercaptoethanol. The washed cells (8 grams) were again collected by centrifugation and resuspended in 20 ml buffer A. The cells were broken by three passes through a French Pressure cell. A 25 µl aliquot of diethylpyrocarbonate was added to inhibit ribonuclease activity. The cell paste was centrifuged at 17.5 K rpm in a JA20 rotor for one hour. The top 2/3 of the supernatant was removed and carefully layered onto an equal volume of sucrose cushion: 20 mM HEPES (pH 7.5), 10.5 mM Mg(OAc)$_2$, 0.5 mM EDTA, 0.5 M NH$_4$Cl, 10 mM β-mercaptoethanol, and 1.1 M sucrose. Ribosomes were pelleted by centrifugation at 40 K rpm for 14 hours in a
Beckman Ti 70 rotor. Purified 70S ribosomes were obtained by washing the crude ribosomes three times with 1.0 M \( \text{NH}_4\text{Cl} \). The ribosome pellet was resuspended in 10 mM Tris-HCl (pH 7.8), 10 mM \( \text{MgCl}_2 \), 1 mM \( \beta \)-mercaptoethanol, and 1.0 M \( \text{NH}_4\text{Cl} \), and shaken gently for 12 hours. \( \text{NH}_4\text{Cl} \) washed ribosomes were pelleted by centrifugation at 40 K rpm for three hours. The \( \text{NH}_4\text{Cl} \) washing procedure was repeated twice. The final ribosome pellet was suspended in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM \( \text{Mg(OAc)}_2 \), 60 mM \( \text{NH}_4\text{Cl} \), and 3 mM \( \beta \)-mercaptoethanol. The final \( A_{260}/A_{280} \) ratio was determined to be 2.0.

**In vitro Poly(U,G)-Directed (Val, Phe) Polypeptide Synthesis**

The *in vitro* polypeptide synthesis reaction was based on the procedure of Ofengand *et al.* (1974). The reaction was carried out in a 100 \( \mu \)l reaction mixture containing 50 mM Bicine (pH 7.5), 50 mM \( \text{NH}_4\text{Cl} \), 20 mM \( \text{Mg(OAc)}_2 \), 3 mM ATP, 1 mM DTT, 1 mM GTP, 10 mM phosphoenol pyruvate (PEP), 0.05 mM unlabeled valine, 0.05 mM unlabeled phenylalanine, 154 pmol of *E. coli* tRNA\(^{\text{Phe}}\), 90 pmol of \( [^3\text{H}] \)valyl-tRNA\(^{\text{Val}} \), 40 \( \mu \)g *E. coli* S-100, 32 \( \mu \)g of poly(U,G), 4 units pyruvate kinase, 3.7 \( A_{260} \) units of \( \text{NH}_4\text{Cl} \) washed ribosomes. Reactions were initiated by addition of 70S ribosomes (ribosomes were pre-incubated at 37°C for 30 minutes), and the reaction mixture was incubated at 37°C for the indicated time intervals. The reaction was stopped by adding 5% TCA, and the mixture was heated at 95°C for 20 minutes. After samples had cooled on ice for 20 minutes, the TCA precipitates were collected on Millipore filters (Millipore Corp.), and washed four times with cold 5% TCA and once with cold 95% ethanol. Filters were dried and the radioactivity was determined by liquid scintillation counting in BioSafe NA scintillation fluor.
Purification of Elongation Factor Tu

*E. coli* JM109 was transformed with pQECluf containing the gene for *T. thermophilus* elongation factor Tu. The purification procedure was a simple one-column step utilizing the presence of a His-tag at the C-terminus of the protein (Ribeiro et al., 1995). A 500 ml culture at 37°C was grown to mid-log phase, induced by adding 1 mM IPTG, and allowed to grow for five more hours. Cells were collected by centrifugation and resuspended in 10 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM DTT, 10 μM PMSF, 1 mM sodium azide, 40 μl of 0.25 M EDTA. Five mg of lysozyme were then added, and the solution was stirred on a magnetic stir-plate for 10 minutes. 200 μl of 4% sodium deoxycholate and 20 μl of 10 mg/ml DNase I were added, and the solution was again stirred for a short time and then left at room temperature for 20 minutes. Cell debris was collected by centrifugation at 30,000x g for 30 minutes, and the supernatant was subject to centrifugation at 100,000x g for 3 hours. The clear supernatant was dialyzed against buffer A: 20 mM potassium phosphate (pH 7.5), 500 mM KCl. The dialyzed sample was loaded on a Ni-NTA Superflow nickel column (Qiagen Inc.) equilibrated with buffer A at a flow rate of 0.5 ml/min. Following loading of the sample, the column was washed with two column volumes of buffer B containing: 20 mM potassium phosphate (pH 6.2), 500 mM KCl. EF-Tu was then eluted with a linear gradient of 0 to 500 mM imidazole in buffer B in 10 column volumes. 2.5 ml fractions were collected, and their A₂₈₀ values measured. Purified elongation factor Tu was homogeneous as determined by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Proteins were visualized by
staining the gels with 1% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid.

**Purification of *E. coli* Phenylalanyl-tRNA Synthetase**

*E. coli* phenylalanyl-tRNA synthetase (PheRS) was partially purified from *E. coli* JM109 based on the procedure of Peterson and Uhlenbeck (Peterson and Uhlenbeck, 1992) from the 35% ammonium sulfate precipitate obtained in the course of preparing ValRS (Chu and Horowitz, 1991). The ammonium sulfate precipitate was resuspended in sample buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA 10% glycerol, and 5 mM β-mercaptoethanol, and dialyzed extensively against the same buffer. A white precipitate formed in the dialysis tube. The supernatant containing PheRS activity was further dialyzed against the same buffer, and then chromatographed on a Mono Q HR 10/10 column (Pharmacia) using the FPLC system. The column was equilibrated in sample buffer containing 40 mM KCl. After loading the dialyzed sample, proteins were eluted with a 200 ml linear gradient of KCl from 40 mM to 400 mM. PheRS activity eluted between 200 mM and 250 mM KCl.

**Determination of Ternary Complex Formation by Hydrolysis Protection Assay**

Interaction of EF-Tu:GTP with aminoacyl-tRNA protects the labile aminoacyl ester bond from spontaneous hydrolysis (Pingoud *et al.*, 1977). Hydrolysis protection assays were performed at 37°C in a 160 µl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl₂, 0.3 µM [³H] aminoacyl-tRNA, and 3 µM EF-Tu:GTP. The pH of the reaction was chosen because it allowed a significant spontaneous hydrolysis to occur in a reasonable amount of time. The reaction was initiated by the addition of [³H] aminoacyl-
tRNA. 20 µl aliquots were removed at the indicated time intervals and spotted on TCA-presoaked Whatman 3 MM filter paper. Filter papers were processed as described for the aminoacylation plateau charging assay, and radioactivity was determined by liquid scintillation counting in BioSafe NA scintillation fluor. EF-Tu:GDP was first activated to EF-Tu:GTP by incubation of 40 µM EF-Tu:GDP at 37°C for three hours in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM KCl, 440 µM GTP, 6 mM PEP, 5 mM DTT, 1 unit/µl pyruvate kinase.

**Ribonuclease Protection Assay to Determine Dissociation Constants of Ternary Complexes**

Dissociation constants of EF-Tu:GTP:valyl-tRNA Val ternary complexes were determined by a RNase protection assay based on the method of Louie and Jurnak (1985). The principle of the assay involves allowing formation of a ternary complex between [³H] valyl-tRNA Val and EF-Tu:GTP. Uncomplexed [³H] valyl-tRNA Val is digested by brief exposure to pancreatic ribonuclease A, and [³H] valyl-tRNA Val associated in a ternary complex is determined by measuring radioactivity that remains TCA precipitable. The assay was performed at two concentrations of [³H] valyl-tRNA Val and seven concentrations of EF-Tu:GTP. Reactions were carried out in 100 µl mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 150 mM NH₄Cl, 5 mM DTT, 3 mM PEP, 20 µM GTP, 50 units/ml pyruvate kinase, and the indicated amount of [³H] valyl-tRNA Val and EF-Tu:GTP. EF-Tu:GDP was activated to EF-Tu:GTP as described for the hydrolysis protection assay. Each reaction mixture was incubated on ice for 15 minutes and then treated with 5 µl of 2 mg/ml pancreatic
ribonuclease A (Sigma) for 15 seconds. Ribonuclease digestion was stopped by rapid
addition of 50 µl of 1mg/ml bulk yeast RNA (Sigma) and 200 µl of cold 10% TCA.
Precipitates were collected on Millipore filters, washed three times with 10% TCA, dried,
and counted by liquid scintillation counting in BioSafe NA scintillation fluor. The
dissociation constant is obtained by employing this equation:

\[
K_d = \frac{[\text{uncomplexed EF-Tu:GTP}][\text{uncomplexed } \left[^{3}\text{H}\right]\text{valyl-tRNA}^{\text{Val}}]}{[\text{EF-Tu:GTP}: \left[^{3}\text{H}\right]\text{valyl-tRNA}^{\text{Val}}]}
\]

In order to obtain an accurate \(K_d\) value, it is essential to determine precisely the
concentration of EF-Tu:GTP and valyl-tRNA\(^{\text{Val}}\) in the reaction. The concentration of EF-
Tu:GTP was determined by titrating a fixed amount of EF-Tu:GTP with increasing amounts
of valyl-tRNA\(^{\text{Val}}\) (Figure 4). In the experiment shown, the fixed amount of EF-Tu:GTP was
10 pmol based on the Bradford assay (Bradford, 1976), and the amount of valyl-tRNA\(^{\text{Val}}\)
protected reached a plateau level at 4 pmol. Assuming a 1:1 EF-Tu:GTP/valyl-tRNA\(^{\text{Val}}\)
complex, 40% of the EF-Tu:GTP in the reaction was active in ternary complex formation.
Typically, the active concentration of EF-Tu:GTP was between 35 to 45% of added EF-
Tu:GTP. To determine the active valyl-tRNA\(^{\text{Val}}\) concentration, a similar approach was
taken. A fixed amount of valyl-tRNA\(^{\text{Val}}\) was titrated with increasing amount of EF-Tu:GTP
(Figure 5). In this experiment, the amount of wild type valyl-tRNA\(^{\text{Val}}\) was fixed at 7 pmol,
and the amount protected at high EF-Tu:GTP concentrations reached a plateau of 6 pmol.
Therefore, about 86% of the valyl-tRNA\(^{\text{Val}}\) was active in ternary complex formation.
Figure 4. Determination of active [EF-Tu:GTP] in the ribonuclease protection assay.

EF-Tu:GTP concentration is fixed at 10 pmole.
Figure 5. Determination of active [valyl-tRNA^{Val}] in the ribonuclease protection assay.

Valyl-tRNA^{Val} concentration is fixed at 7 pmole.
RESULTS

Search for the Complete Identity Elements in \textit{E. coli} tRNA^{Val}

Two different approaches were used to search for the complete identity elements in \textit{E. coli} tRNA^{Val}. First, the aminoacylation efficiency of \textit{in vitro} transcribed tRNA^{Val} variants, obtained by introducing specific nucleotide changes into wild-type tRNA^{Val}, was determined. When the catalytic efficiency for a tRNA^{Val} variant is significantly reduced, it can be attributed to the absence of recognition nucleotide(s) in that tRNA. With this approach, our laboratory has identified several identity elements in \textit{E. coli} tRNA^{Val} (Chu \textit{et al.} 1992; Liu, 1995). Second, identity swap experiments were carried out to identify positive identity elements essential for ValRS recognition and negative identity elements which prevent proper recognition by ValRS. In these experiments, identity determinants of tRNA^{Val} were introduced into the sequence framework of another tRNA, and the chimeric tRNA was tested for the efficiency of its aminoacylation with valine. Liu (1995) has successfully converted \textit{E. coli} tRNA^{AUA}(UGC) to an efficient substrate for ValRS. Building on the success of these experiments, the goal of this study is to understand all the determinants that govern the specific and efficient recognition of \textit{E. coli} tRNA^{Val} by ValRS.

\textbf{Kinetic Study of \textit{E. coli} tRNA^{Val} Variants}

It was previously found in our laboratory and others that anticodon positions A35 and C36 are the major positive synthetase recognition determinants of tRNA^{Val} (Schulman and Pelka, 1988; Tamura \textit{et al.}, 1991; Chu \textit{et al.}, 1992). Any mutation introduced into these two positions either completely abolishes or greatly reduces the aminoacylation activity of
tRNA\textsuperscript{Val} variants. In addition, the discriminator base A73 (Liu, 1995; Tamura \textit{et al.}, 1991) and position G20 (Liu, 1995) in the D-loop were identified as recognition nucleotides of tRNA\textsuperscript{Val}.

Aminoacylation kinetic studies of tRNA\textsuperscript{Val} variants were carried out to identify additional determinants essential for valyl-tRNA synthetase (ValRS) recognition of the tRNA, and to detect negative determinants which prevent proper/efficient recognition by ValRS. These studies help us to understand the complexity of tRNA-synthetase interactions and how the high specificity of aminoacylation is achieved.

\textit{E. coli} tRNA\textsuperscript{Val} variants in the acceptor stem

The acceptor stem contains major recognition nucleotides for some tRNAs (Hou and Schimmel, 1988; McClain and Foss, 1988; Jahn \textit{et al.}, 1991; Liu \textit{et al.}, 1995). The premier example is tRNA\textsuperscript{Ala}. Its identity element has been found to reside almost exclusively in a single G3:U70 base pair by both \textit{in vivo} (McClain and Foss, 1988) and \textit{in vitro} (Hou and Schimmel, 1988) experiments. Tamura and coworkers reported that changing the base pair U4:A69 to A4:U69 in tRNA\textsuperscript{Val} decreases the aminoacylation activity of tRNA\textsuperscript{Val} by 5.3-fold, and substitution of G3:C70 with C3:G70 reduces the activity of tRNA\textsuperscript{Val} by 2.3-fold (Tamura \textit{et al.}, 1991). They concluded that both the U4:A69 and the G3:C70 base pairs are identity elements of tRNA\textsuperscript{Val}. Results from our laboratory contradict these conclusions (Liu, 1995). Liu (1995) found that the same mutations do not reduce the aminoacylation activity of the tRNA\textsuperscript{Val} significantly; the C4:G69 variant of tRNA\textsuperscript{Val} was also a good substrate for ValRS (Vahid Feiz, unpublished results). To complete the set of Watson Crick base pair...
Table 1. Kinetic parameters for aminoacylation of tRNA\textsuperscript{Val} variants at position 4:69 in the acceptor stem

<table>
<thead>
<tr>
<th>tRNA\textsuperscript{Val} Variants</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmol/min/mg)</th>
<th>$V_{max}/K_m$</th>
<th>Relative ($V_{max}/K_m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA\textsuperscript{Val}</td>
<td>1.4</td>
<td>5.0</td>
<td>3.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>U4:A69 → G4:C69</td>
<td>6.2</td>
<td>6.8</td>
<td>1.1</td>
<td>0.31</td>
</tr>
<tr>
<td>U4:A69 → G4:U69</td>
<td>20.0</td>
<td>1.9</td>
<td>0.095</td>
<td>0.026</td>
</tr>
<tr>
<td>U4:A69 → U4:G69</td>
<td>10.4</td>
<td>6.2</td>
<td>0.60</td>
<td>0.17</td>
</tr>
<tr>
<td>U4:A69 → U4*C69</td>
<td>2.9</td>
<td>5.8</td>
<td>2.0</td>
<td>0.56</td>
</tr>
<tr>
<td>U4:A69 → A4*G69</td>
<td>8.9</td>
<td>6.1</td>
<td>0.69</td>
<td>0.19</td>
</tr>
<tr>
<td>A6:U67 → G6:U67</td>
<td>3.2</td>
<td>5.9</td>
<td>1.8</td>
<td>0.50</td>
</tr>
<tr>
<td>U7:A66 → U7:G66</td>
<td>4.1</td>
<td>7.2</td>
<td>1.8</td>
<td>0.50</td>
</tr>
</tbody>
</table>

substitutions at position 4:69, G4:C69 was constructed. Both $K_m$ and $V_{max}$ are increased by 4.4-fold and 1.4-fold, respectively, resulting in a 3.2-fold decrease in the catalytic efficiency (Table 1). Introduction of G4:C69 into the acceptor stem of tRNA\textsuperscript{Val} convert the first five base pairs into a series of consecutive G:C base pairs (Figure 2), and this may contribute to
the lower activity of this mutant (see Discussion). Thus Watson-Crick base pair substitutions at position 4:69 of *E. coli* tRNA^Val^ have little effect on the aminoacylation activity of tRNA^Val^.

Watson-Crick base pair substitutions at other positions in the acceptor stem helix also do not significantly affect aminoacylation (Liu, 1995). However, introduction of G:U wobble base pair substitutions in the first three base pairs of the acceptor stem helix lead to a gradual decrease in activity as the site of mutation moves closer to position 4:69 (Liu, 1995). To further investigate the effects of G:U base pairs in the acceptor helix of tRNA^Val^ on aminoacylation activity, G:U base pairs were introduced into the bottom half of the acceptor stem (4:69, 6:67, and 7:66). Introduction of wobble base pairs G4:U69 or U4:G69 results in a large reduction, 38 and 6-fold respectively, in $V_{max}/K_m$ (Table 1). Loss of activity is due mainly to a large increase in $K_m$ in these two wobble base pair variants of tRNA^Val^. A significant decrease in $V_{max}$ is also observed for the G4:U69 variant. Wobble base pair (G:U) substitutions into position 6:67 or 7:66, the last two base pairs of the acceptor stem, have only moderate effects on the catalytic efficiency of tRNA^Val^ (Table 1).

Two additional non-canonical base pairs, U:C and A:G, were introduced at position 4:69. The U4:C69 mutant tRNA has decent activity, 56% that of the wild type tRNA (Table 1). Aminoacylation activity of the A4:G69 tRNA^Val^ variant is considerably lower, 19% of wild type activity (Table 1). These results indicate that a pyrimidine:pyrimidine mismatch is tolerated better than a purine:purine mismatch (see Discussion).
Effect of acceptor stem variants of tRNA$^\text{Val}$ on the affinity for valine in aminoacylation

Recently, Söll and coworkers showed that for some aminoacyl-tRNA synthetases interaction with identity determinants in tRNA affects the amino acid affinity of the enzyme, and this may influence identification of synthetase recognition determinants in the tRNA (Ibba et al., 1996; Hong et al., 1996). Recognition determinants of *E. coli* tRNA$^\text{Gln}$ were first identified by aminoacylation kinetic studies carried out at sub-optimal amino acid concentration (Jahn et al., 1991). Several of these were later reclassified when optimal amino acid concentrations were used.

Steady-state kinetic studies were conducted with wild type *E. coli* tRNA$^\text{Val}$ and with several acceptor stem variants to investigate the effects of base substitutions on the affinity of ValRS for valine in the aminoacylation reaction. Michaelis constants ($K_m$) for valine in the aminoacylation reaction were determined. The $K_m$ value for valine in the reaction with wild type tRNA$^\text{Val}$ is 42.8 μM (Table 2); this value is similar to the value of 45 μM previously determined (Chu and Horowitz, unpublished). The $V_{\text{max}}$ for this reaction was found to be 6.50 μmol/min/mg, which is slightly higher than the $V_{\text{max}}$ value of 5.0 reported for wild type tRNA$^\text{Val}$ in Table 1. In aminoacylation of the A4:U69 tRNA$^\text{Val}$ variant, $K_m$ and $V_{\text{max}}$ values of 45.9 and 5.84, respectively, were determined (Table 2). Both values are comparable to those of the reaction with wild type tRNA$^\text{Val}$. The $K_m$ and $V_{\text{max}}$ values observed with C3:G70 tRNA$^\text{Val}$ are slightly higher than those with wild type tRNA (Table 2). Overall, wild type tRNA$^\text{Val}$ and the two tRNA$^\text{Val}$ variants in the acceptor stem have comparable $V_{\text{max}}/K_m$ values. These results suggest that at least several mutations in the acceptor stem of tRNA$^\text{Val}$ do not influence the valine affinity of ValRS.
Table 2. Kinetic parameters for aminoacylation of tRNA\textsuperscript{Val} variants in the acceptor stem:

Determination of K_\text{m} for valine

<table>
<thead>
<tr>
<th>tRNA\textsuperscript{Val} Variants</th>
<th>K_\text{m} (\mu M) (valine)</th>
<th>\text{V}_{\text{max}} (\mu mol/min/mg)</th>
<th>\text{V}<em>{\text{max}}/K</em>\text{m}</th>
<th>Relative (\text{V}<em>{\text{max}}/K</em>\text{m})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA\textsuperscript{Val}</td>
<td>42.8</td>
<td>6.50</td>
<td>0.15</td>
<td>(1.0)</td>
</tr>
<tr>
<td>U4:A69 → A4:U69</td>
<td>45.9</td>
<td>5.84</td>
<td>0.13</td>
<td>0.87</td>
</tr>
<tr>
<td>G3:C70 → C3:G70</td>
<td>59.8</td>
<td>7.18</td>
<td>0.12</td>
<td>0.80</td>
</tr>
</tbody>
</table>

The kinetic parameters for aminoacylation of tRNA\textsuperscript{Val} variants were previously determined at valine concentration of 100 \mu M (about 2 times the K_\text{m} for valine in the reaction with wild type tRNA\textsuperscript{Val} and the two acceptor stem variants). Kinetic parameters for aminoacylation of tRNA\textsuperscript{Val} variants were also measured at valine concentrations of 10 \mu M, below their K_\text{m} (sub-optimal concentration), to determine whether mutations in the acceptor stem and discriminator base (position 73) affect the substrate efficiency of tRNA\textsuperscript{Val} under these conditions. Wild type tRNA\textsuperscript{Val} has K_\text{m} and \text{V}_{\text{max}} values of 0.79 and 1.19, respectively, at 10 \mu M valine (Table 3). K_\text{m} and \text{V}_{\text{max}} for the A4:U69 variant are very close to those for wild type tRNA\textsuperscript{Val}; it has a catalytic efficiency 83\% that of the wild-type (Table 3). The C3:G70 tRNA\textsuperscript{Val} variant has slightly elevated K_\text{m} and \text{V}_{\text{max}} values that result in a relative catalytic efficiency of 72\%. These results agree well with our previous results, obtained at
Table 3. Kinetic parameters for aminoacylation of tRNA<sup>Val</sup> variants in the acceptor stem under sub-optimal valine concentration (10 μM)

<table>
<thead>
<tr>
<th>tRNA&lt;sup&gt;Val&lt;/sup&gt; Variants</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM) (tRNA)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (μmol/min/mg)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Relative (V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA&lt;sup&gt;Val&lt;/sup&gt;</td>
<td>0.79</td>
<td>1.19</td>
<td>1.51</td>
<td>(1.0)</td>
</tr>
<tr>
<td>U4:A69 → A4:U69</td>
<td>0.77</td>
<td>0.97</td>
<td>1.26</td>
<td>0.83</td>
</tr>
<tr>
<td>G3:C70 → C3:G70</td>
<td>1.27</td>
<td>1.37</td>
<td>1.08</td>
<td>0.72</td>
</tr>
<tr>
<td>A73 → C73</td>
<td>0.74</td>
<td>0.71</td>
<td>0.96</td>
<td>0.64</td>
</tr>
<tr>
<td>A73 → U73</td>
<td>1.46</td>
<td>0.90</td>
<td>0.62</td>
<td>0.41</td>
</tr>
<tr>
<td>A73 → G73</td>
<td>---</td>
<td>---</td>
<td>0.011</td>
<td>0.007</td>
</tr>
</tbody>
</table>

100 μM valine, that showed A4:U69 and C3:G70 variants having 86% and 81% of wild type tRNA<sup>Val</sup> aminoacylation activity (Liu, 1995) and indicate that U4:A69 and G3:C70 are not identity elements in tRNA<sup>Val</sup> for recognition by ValRS or for specific valine recognition.

Similar experiments were carried out with tRNA<sup>Val</sup> having nucleotide substitutions at position 73. The discriminator base, A73, of tRNA<sup>Val</sup> was previously found to be an identity element because substitution by G reduces activity in the aminoacylation reaction 38-fold at 100 μM valine (Liu, 1995); the U73 and C73 variants are, respectively, 31% and 44% as active as wild-type tRNA<sup>Val</sup> (A73). Substitution of A73 with either C73 or U73 results in
tRNA\textsuperscript{Val} variants that have a moderate decrease in activity at 10 \mu M valine (Table 3). The C73 tRNA\textsuperscript{Val} variant is 64% as active as wild type (Table 3); the decrease is due entirely to a 1.7 fold decrease in $V_{\text{max}}$. For the U73 tRNA\textsuperscript{Val} variant, the activity loss comes from a 1.8 fold increase in $K_m$ and a 1.3 fold decrease in $V_{\text{max}}$; its activity is 41% that of wild-type tRNA\textsuperscript{Val} (Table 3). The activity of the G73 tRNA\textsuperscript{Val} variant is lower than that of wild-type tRNA\textsuperscript{Val} by two orders of magnitude (Table 3). The individual $K_m$ and $V_{\text{max}}$ values were not determined due to the low activity. Values for the relative specificity constants agree well with our previous results, obtained at 100 \mu M valine.

**Mutation of nucleotides involved in tertiary interactions**

The role of tertiary interactions in maintaining the native folding of tRNAs is well established. Recently, studies with some tRNAs have identified bases involved in tertiary interactions as recognition nucleotides (Liu et al., 1995; Peterson and Uhlenbeck, 1992). In this section, the role of several tertiary interactions in the recognition of tRNA\textsuperscript{Val} by ValRS is investigated further. The following studies focus on positions 26, 44, and 45 of *E. coli* tRNA\textsuperscript{Val}.

In the crystal structure of yeast tRNA\textsuperscript{Phe} bases 26 and 44 form a purine-purine base pair at the junction of the anticodon and D stems. This base pair is stacked on both stems, and is responsible for a 26° kink between these two helices (Kim, 1976). An adenine (A) or guanine (G) occurs at position 26 in 95% of *E. coli* tRNAs. Therefore, A26 was first substituted with G26; and the result indicates that the $V_{\text{max}}/K_m$ of the G26 tRNA\textsuperscript{Val} variant is 45% that of wild type tRNA\textsuperscript{Val} (Table 4). A base change at position 44 results in a slightly
Table 4. Kinetic parameters for aminoacylation of tRNA^{Val} variants involved in tertiary interaction

<table>
<thead>
<tr>
<th>tRNA^{Val} Variants</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmol/min/mg)</th>
<th>$V_{max}/K_m$</th>
<th>Relative $(V_{max}/K_m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA^{Val}</td>
<td>1.4</td>
<td>5.0</td>
<td>3.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>A26 $\rightarrow$ G26</td>
<td>3.6</td>
<td>5.8</td>
<td>1.6</td>
<td>0.45</td>
</tr>
<tr>
<td>G44 $\rightarrow$ A44</td>
<td>3.5</td>
<td>4.4</td>
<td>1.3</td>
<td>0.36</td>
</tr>
<tr>
<td>A26-G44 $\rightarrow$ G26-A44</td>
<td>1.9</td>
<td>7.1</td>
<td>3.7</td>
<td>1.04</td>
</tr>
<tr>
<td>G45 $\rightarrow$ A45</td>
<td>3.8</td>
<td>6.6</td>
<td>1.7</td>
<td>0.48</td>
</tr>
<tr>
<td>G45 $\rightarrow$ U45</td>
<td>2.7</td>
<td>5.5</td>
<td>2.1</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Greater reduction of aminoacylation activity to 36% that of the wild-type (Table 4). In both cases, the loss of aminoacylation activity is due largely to an increase in $K_m$ by about 2.5-fold.

A double mutant was constructed by replacing A26 with G26 and G44 with A44. These substitutions recapitulate the A:G tertiary base pair, although the locations of the bases are interchanged. This double mutant, G26:A44 tRNA^{Val}, is as good a substrate for valyl-tRNA synthetase as wild type tRNA^{Val} (Table 4).

Position 45 is involved in a tertiary interaction with base pair 10:25 in the D-stem (see Figure 1A). Substitution of G45 by A reduces the specificity constant ($V_{max}/K_m$) of the tRNA^{Val} variant 2.1-fold compared to wild type tRNA^{Val} (Table 4). Replacement of G45 by
U45 reduces the specificity constant 1.8-fold (Table 4). In both tRNA$^{\text{Val}}$ variants, the $V_{\text{max}}$ remains relatively unchanged compared to that of wild type tRNA$^{\text{Val}}$; the reduction in the specificity constant is due mainly to an increase in $K_m$.

**Identity Swap Experiments**

The accuracy of aminoacylation reactions depend not only on the efficient and productive interaction of a tRNA molecule with its cognate aminoacyl-tRNA synthetase, but also on how the synthetase discriminates against noncognate tRNA molecules. To further investigate the structural requirements for the recognition of *E. coli* tRNA$^{\text{Val}}$ by ValRS, and to uncover possible negative determinants and/or additional recognition nucleotides, identity swap experiments were carried out by transplanting the known recognition elements of tRNA$^{\text{Val}}$ into yeast tRNA$^{\text{Phe}}$ and *E. coli* tRNA$^{\text{Phe}}$.

**Conversion of yeast tRNA$^{\text{Phe}}$ to a valine acceptor**

Nucleotide substitution in *E. coli* tRNA$^{\text{Val}}$ had shown that A35, C36, A73 and to a lesser extent G20 are recognition determinants for ValRS. If this set of synthetase recognition nucleotides in tRNA$^{\text{Val}}$ is complete, then it is reasonable to postulate that introduction of these four recognition nucleotides into noncognate tRNA molecules will convert them to valine accepting species. Figure 4 compares the sequence of *E. coli* tRNA$^{\text{Val}}$ and yeast tRNA$^{\text{Phe}}$. Yeast tRNA$^{\text{Phe}}$ differs from tRNA$^{\text{Val}}$ at 28 positions (shown boxed in Figure 6), most of which are located in the acceptor stem, anticodon stem and loop, and T-stem. Yeast tRNA$^{\text{Phe}}$ has three of the four identified tRNA$^{\text{Val}}$ recognition nucleotides; only position 36 differs, tRNA$^{\text{Phe}}$ has A in place of the C36 in tRNA$^{\text{Val}}$. The recognition model predicts that changing A36 of yeast tRNA$^{\text{Phe}}$ to C36 should result in an efficient substrate for
Figure 6. Comparison of the nucleotide sequences of *E. coli* tRNA\(^{Val}\) (UAC anticodon) and yeast tRNA\(^{Phe}\) (GAA anticodon).

Shaded regions indicate sequence differences between the two tRNAs.
E. coli tRNA\textsuperscript{Val} (UAC)

Yeast tRNA\textsuperscript{Phe} (GAA)
ValRS.

Wild type yeast tRNA$^{\text{Phc}}$(GAA) is a very poor substrate for ValRS. Its level of aminoacylation with valine is almost undetectable, its specificity constant is at least five orders of magnitude lower than that for wild type E. coli tRNA$^{\text{Val}}$ (Table 5). Because C36 is a major identity element for tRNA$^{\text{Val}}$, substitution of A36 with C36 in yeast tRNA$^{\text{Phc}}$, as expected, markedly increases the valine accepting efficiency of yeast tRNA$^{\text{Phc}}$ (Table 5). However, this yeast tRNA$^{\text{Phc}}$(GAC) variant still has only 14% of the wild type tRNA$^{\text{Val}}$ activity.

Table 5. Kinetic parameters for aminoacylation of yeast tRNA$^{\text{Phc}}$ variants with valine

<table>
<thead>
<tr>
<th>tRNA$^{\text{Val}}$ Variants</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmol/min/mg)</th>
<th>$V_{max}/K_m$</th>
<th>Relative $(V_{max}/K_m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA$^{\text{Val}}$</td>
<td>1.4</td>
<td>5.0</td>
<td>3.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>tRNA$^{\text{Phc}}$(GAA)</td>
<td>----</td>
<td>----</td>
<td>3.9x10$^{-5}$</td>
<td>1.1x10$^{-5}$</td>
</tr>
<tr>
<td>tRNA$^{\text{Phc}}$(GAC)</td>
<td>3.3</td>
<td>1.7</td>
<td>0.52</td>
<td>0.14</td>
</tr>
<tr>
<td>tRNA$^{\text{Phc}}$(GAC) A4</td>
<td>2.1</td>
<td>4.15</td>
<td>2.0</td>
<td>0.56</td>
</tr>
<tr>
<td>tRNA$^{\text{Phc}}$(GAA) A4</td>
<td>----</td>
<td>----</td>
<td>1.3x10$^{-4}$</td>
<td>3.6x10$^{-5}$</td>
</tr>
</tbody>
</table>
Previous experiments showed that a G4:U69 wobble base pair in the acceptor stem of tRNA\textsuperscript{Val} reduces the valine accepting activity of the tRNA considerably (Table 1). The G:U pair at position 4:69 in the acceptor stem of yeast tRNA\textsuperscript{Phe} could be the reason that yeast tRNA\textsuperscript{Phe}(GAC), which has all the recognition nucleotides of tRNA\textsuperscript{Val}, is a poor substrate for ValRS.

When the G4:U69 base pair of yeast tRNA\textsuperscript{Phe}(GAC) is converted to an A4:U69 Watson-Crick base pair by mutation of G4 to A4, the aminoacylation activity of the resulting tRNA increases considerably. This tRNA\textsuperscript{Phe} variant has a relative $V_{\text{max}}/K_m$ of 0.56 compared to that of wild type tRNA\textsuperscript{Val} (Table 5). Changing G4:U69 to A4:U69 alone, in the absence of the A36 to C36 change, allows yeast tRNA\textsuperscript{Phe} to accept valine slightly better than wild type yeast tRNA\textsuperscript{Phe}, but its relative $V_{\text{max}}/K_m$ is still almost five orders of magnitude lower than that of tRNA\textsuperscript{Val} (Table 5). These results show that two nucleotide changes in yeast tRNA\textsuperscript{Phe}, A36 to C36 and elimination of the G:U wobble base pair in the acceptor stem, convert the tRNA into a good valine acceptor.

Conversion of \textit{E. coli} tRNA\textsuperscript{Phe} to a valine acceptor

A similar identity swap experiment was carried out with \textit{E. coli} tRNA\textsuperscript{Phe} to examine the effects of the known recognition determinants in another tRNA sequence framework. The sequence of \textit{E. coli} tRNA\textsuperscript{Phe} differs from that of \textit{E. coli} tRNA\textsuperscript{Val} at 32 positions, including most of the acceptor stem, the entire anticodon stem, part of the anticodon loop and part of the T-stem (Figure 7). \textit{E. coli} tRNA\textsuperscript{Phe} has two of the four tRNA\textsuperscript{Val} recognition nucleotides, A35 and A73, but has A36 in place of C36 and U20 instead of G20. The
Figure 7. Comparison of the nucleotide sequences of *E. coli* tRNA\textsuperscript{Val} (UAC anticodon) and *E. coli* tRNA\textsuperscript{Phe} (GAA anticodon).

Shaded regions indicate sequence differences between the two tRNAs.
E. coli tRNA^{Val} (UAC)

E. coli tRNA^{Phe} (GAA)
recognition model predicts that changing U20 and A36 of *E. coli* tRNA\textsuperscript{Phe} to G20 and C36 should result in an efficient substrate for ValRS.

Wild type *E. coli* tRNA\textsuperscript{Phe}(GAA) is not a substrate for ValRS (Table 6). Substitution of U20 and A36 in *E. coli* tRNA\textsuperscript{Phe} with G20 and C36 allows this variant to accept valine at a low level (see also Liu, 1995). The catalytic efficiency of this tRNA\textsuperscript{Phe} variant is only 1%

| Table 6. Kinetic parameters for aminoacylation of acceptor stem and variable loop mutants |
|-----------------------------------------------|--------------|---------------|----------------|----------------|
| *E. coli* tRNA\textsuperscript{Phe} with valine |
| tRNA\textsuperscript{Val} Variants             | K\textsubscript{m} (µM) | V\textsubscript{max} (µmol/min/mg) | V\textsubscript{max}/K\textsubscript{m} | Relative (V\textsubscript{max}/K\textsubscript{m}) |
| Wild type tRNA\textsuperscript{Val}            | 1.4          | 5.0          | 3.6          | (1.0)         |
| tRNA\textsuperscript{Phe}(GAA)                 | No Detectable Aminoacylation |
| tRNA\textsuperscript{Phe}(GAC) G20             | 17.1         | 0.58         | 0.03         | 0.01          |
| tRNA\textsuperscript{Phe}(GAC) G20 M3\textsuperscript{*} | 14.7         | 3.4          | 0.23         | 0.06          |
| tRNA\textsuperscript{Phe}(GAC) G20M3 A6:U67    | 7.2          | 3.4          | 0.47         | 0.13          |
| tRNA\textsuperscript{Phe}(GAC) G20M3 G6:U67    | 11.0         | 2.1          | 0.19         | 0.05          |
| tRNA\textsuperscript{Phe}(GAC) G20 G45        | 23.3         | 2.1          | 0.09         | 0.03          |
| tRNA\textsuperscript{Phe}(GAC) G20 M3 G45     | 8.0          | 5.0          | 0.63         | 0.18          |

\textsuperscript{*} M3 represents the three base pair mutations of C27:G43, C28:G42, and U29:A41.
that of wild type tRNA^{Val} (Table 6). Additional mutations were introduced into this tRNA to improve its efficiency as a substrate for ValRS and to identify additional positive and/or negative recognition nucleotides.

**tRNA^{Phe} variants in the acceptor stem**

The acceptor stem of wild type tRNA^{Phe} (GAA) is very rich in G:C base pairs in its (Figure 7), six of the seven base pairs are G:C/G:C base pairs. The aminoacylation of tRNA^{Phe} variants with valine may be affected by the G:C-rich acceptor stem helix. To decrease the number of G:C base pairs in the acceptor stem helix, the G6:C67 base pair of *E. coli* tRNA^{Phe}(GAC) G20 was mutated by making a single base substitution that changes base pair G6:C67 to G6:U67, and a two-base substitution that changes base pair G6:C67 to A6:U67. The velocity of aminoacylation of these tRNA^{Phe} variants is shown on Figure 8. The double mutant has an aminoacylation rate that is slightly higher than tRNA^{Phe} variants without the mutation, while the base substitution that introduces a G:U base pair into the acceptor stem causes a small but consistent decrease in the rate of aminoacylation. The same mutations at base pair 6:67 were also introduced into a variant of *E. coli* tRNA^{Phe}(GAC) G20, tRNA^{Phe}(GAC) G20 M3, which has the top three base pairs of the anticodon stem converted to those found in *E. coli* tRNA^{Val}. Introduction of the M3 mutation into *E. coli* tRNA^{Phe}(GAC) G20 interrupts a series of G:C base pairs in the anticodon stem (Figure 7), but does not significantly improve aminoacylation with valine (Table 6). The time course of aminoacylation of the two tRNA^{Phe}(GAC) G20 M3 variants with mutations at positions 6 and 67 (Figure 9) gave results similar to those found with the same mutations in the framework of tRNA^{Phe}(GAC) G20 (Figure 8). The kinetic parameters for aminoacylation of
Figure 8. Time course of aminoacylation with valine of wild type tRNA\textsuperscript{Val},
tRNA\textsuperscript{Phe}(GAC)G20 A6:U67 (open triangles), tRNA\textsuperscript{Phe}(GAC)G20 (open circles),
and tRNA\textsuperscript{Phe}(GAC)G20 U67 (closed triangles). ValRS and tRNA concentrations
are 0.1 ng/\mu{l} and 2 \mu{M}, respectively.
Figure 9. Time course of aminoacylation with valine of wild type tRNA$^{\text{Val}}$ (closed circles), tRNA$^{\text{Phe}}$ (GAC)G20 M3 A6:U67 (open triangles), tRNA$^{\text{Phe}}$ (GAC)G20 M3 (open circles), and tRNA$^{\text{Phe}}$ (GAC)G20 M3 U67 (closed triangles). ValRS and tRNA concentrations are 0.1 ng/μl and 2 μM, respectively.
tRNA\textsubscript{Phe}(GAC) G20 M3 variants with base substitutions at position 6 and 67 are shown in Table 6. For the double mutant tRNA\textsubscript{Phe}(GAC) G20 M3 A6:U67, \(K_m\) is reduced 2 fold compared to tRNA\textsubscript{Phe}(GAC) G20 M3 while \(V_{\text{max}}\) stays the same (Table 6). For tRNA\textsubscript{Phe}(GAC) G20 M3 U67, the presence of a G:U base pair in the acceptor stem slightly reduces its aminoacylation activity (Table 6). Substitution of A6:U67 with G6:U67 in tRNA\textsubscript{Val} also causes a 2-fold decrease in activity (Table 1).

To disrupt the series of G:C base pairs in the acceptor stem helix, two additional acceptor stem mutants in the framework of \textit{E. coli} tRNA\textsubscript{Phe}(GAC) G20 M3 were constructed: the G3:C70 and C4:G69 base pairs were mutated to A3:U70 and U4:A69, respectively. Aminoacylation rates of these tRNA\textsubscript{Phe} variants were determined and the results are shown in Figure 10. Both these acceptor stem mutants are aminoacylated at a slightly higher rate than tRNA\textsubscript{Phe}(GAC) G20 M3. However, they are still relatively poor substrates for ValRS compared to wild type tRNA\textsubscript{Val}. The determinant(s) that can improve the aminoacylation activity of tRNA\textsubscript{Phe}(GAC) G20 does not appear to be located in the acceptor stem.

\textit{tRNA\textsubscript{Phe} variants in the variable pocket and variable loop}

Positions outside of the acceptor stem were examined for their effects as positive or negative determinants of ValRS recognition. Comparison of the sequence of \textit{E. coli} tRNA\textsubscript{Val} and \textit{E. coli} tRNA\textsubscript{Phe} (Figure 7) shows that in the D-loop, T-loop, and variable regions, there are a total of five sequence differences between the two tRNAs; these are at positions 16, 17, 20, 45 and 60. Four of the five nucleotides (positions 16, 17, 20, and 60) are in the variable
Figure 10. Time course of aminoacylation with valine of wild type tRNA^{Val} (closed circles),
tRNA^{Phe}(GAC)G20 M3 U4:A69 (open triangles), tRNA^{Phe}(GAC)G20 M3 A3:U70 (closed triangles), and tRNA^{Phe}(GAC)G20 (open circles). ValRS and tRNA concentrations are 0.1 ng/μl and 2 μM, respectively.
pocket (Ladner et al., 1975), formed by the tertiary interaction between bases in the D-loop and T-loop. Position 20 has already been mutated because it is a minor recognition element previously identified in tRNA\textsuperscript{Val} (Liu, 1995). The bases at position 16 and 60 of tRNA\textsuperscript{Phe}(GAC) G20 were converted to those present at these positions in tRNA\textsuperscript{Val} to investigate their effect on aminoacylation. Individual substitution of U16 and U60 in tRNA\textsuperscript{Phe} with C16 and C60, respectively, failed to increase valine acceptance. The time course of aminoacylation of C16 and C60 variants are shown on Figure 11. A double mutant of C16 and C60 is also a poor substrate for ValRS (Figure 11).

U45 in the variable loop of tRNA\textsuperscript{Phe} is involved in a tertiary interaction with G10:C25 (see Figure 1). Substitution of U45 in both tRNA\textsuperscript{Phe}(GAC) G20 and tRNA\textsuperscript{Phe}(GAC) G20 M3 by G45 resulted in a consistent 3-fold increase in activity (Table 6). tRNA\textsuperscript{Phe}(GAC) G20 G45 M3 variant has 18% of wild type activity, an 18-fold increase over the tRNA\textsuperscript{Phe} mutant without G45 and M3. The guanine at position 45 appears to modulate aminoacylation activity in the tRNA\textsuperscript{Val}-ValRS interaction.

tRNA\textsuperscript{Phe} variants in the anticodon stem and loop

Previous results from our laboratory indicated that mutation of U29:A41 in the anticodon stem of tRNA\textsuperscript{Val} to C29:G41 results in a 45-fold decrease in aminoacylation activity (Liu, 1995). The decrease in activity is due mainly to a 18-fold increase in \( K_m \). The C29:G41 mutant with five C:G base pairs in the anticodon stem (Figure 2) has a structural resemblance to \textit{E. coli} tRNA\textsuperscript{Phe} which has four consecutive G:C base pairs in the anticodon stem (Figure 7). This could be the structural feature that is preventing \textit{E. coli} tRNA\textsuperscript{Phe}, complete with all the recognition nucleotides of tRNA\textsuperscript{Val}, from being efficiently
Figure 11. Time course of aminoacylation with valine of wild type tRNA^{Val} (closed circles),
tRNA^{Phe}(GAC)G20 C16 (open triangles), tRNA^{Phe}(GAC)G20 C60 (open circles),
and tRNA^{Phe}(GAC)G20 C16 C60 (closed triangles). ValRS and tRNA
concentrations are 0.1 ng/μl and 2 μM, respectively.
aminoacylated by ValRS.

Single base pair mutations in the upper part of anticodon stem, which introduce
sequences found in tRNA$^{\text{Val}}$ into tRNA$^{\text{Phe}}$(GAC) G20 one base pair at a time, were
constructed (Figure 12). Individual base pair substitutions at position 27:43, 28:42, and
29:41 only slightly increase catalytic efficiency (Table 7). The activity increases come from
increases in $V_{\text{max}}$ of 3- to 7-fold. Conversion of all three base pairs at the top of the
anticodon stem to those of tRNA$^{\text{Val}}$ yielded a mutant tRNA$^{\text{Phe}}$, tRNA$^{\text{Phe}}$(GAC) G20 M3
(Figure 12), which also has only a small increase in activity over the tRNA$^{\text{Phe}}$ variant without
M3 (Table 7). The increase in activity is largely due to a 5.9 fold increase in $V_{\text{max}}$. Overall,
the individual base pair or combination of base pair mutations at the top of the anticodon
stem do not significantly increase the activity of $E. \ coli$ tRNA$^{\text{Phe}}$. All variants have a much
higher $K_m$ (10 to 18-fold increase) which indicates that the binding of the substrate to ValRS
is severely affected.

In an attempt to identify additional structural feature(s) that are missing as recognition
nucleotide(s) and/or acting as negative determinant(s) in $E. \ coli$ tRNA$^{\text{Phe}}$(GAC) G20, the
entire anticodon stem and loop sequence of $E. \ coli$ tRNA$^{\text{Phe}}$ was mutated to match the
sequence of tRNA$^{\text{Val}}$. The resulting molecule, tRNA$^{\text{Phe}}$(GAC)(V-stem, V-loop) G20 (Figure
12), has a relative $V_{\text{max}}/K_m$ of 0.36 compared to that of wild type tRNA$^{\text{Val}}$ (Table 7). The
$V_{\text{max}}$ is close to that of the wild-type tRNA$^{\text{Val}}$, and the $K_m$ is only elevated 3-fold. Since G45
was found to modulate the activity of tRNA$^{\text{Val}}$ (Table 6), U45 in tRNA$^{\text{Phe}}$(GAC)(V-stem, V-
loop) G20 was changed to G45. This tRNA$^{\text{Phe}}$ variant is now as active as wild type tRNA$^{\text{Val}}$
(Table 7). The presence of G45 increases the activity almost 3-fold.
Figure 12. Variants of *E. coli* tRNA$^{\text{Phe}}$(GAC) G20 in the anticodon stem and loop. Shaded regions indicate sequence that was mutated to match the sequence found in tRNA$^{\text{Val}}$. 
Table 7. Kinetic parameters for aminoacylation of *E. coli* tRNA^{Phe} variants in the anticodon stem and loop with valine

<table>
<thead>
<tr>
<th>tRNA^{Val} Variants</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) ((\mu)mol/min/mg)</th>
<th>(V_{max}/K_m)</th>
<th>Relative ((V_{max}/K_m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type tRNA^{Val}</td>
<td>1.4</td>
<td>5.0</td>
<td>3.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) G20</td>
<td>17.1</td>
<td>0.58</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) G20 C27:G43</td>
<td>24.8</td>
<td>1.8</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) G20 C28:G42</td>
<td>18.4</td>
<td>4.2</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) G20 U29:A41</td>
<td>22.1</td>
<td>1.5</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) G20 M3 *</td>
<td>14.7</td>
<td>3.4</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) (V-stem,V-loop) G20</td>
<td>4.3</td>
<td>5.6</td>
<td>1.3</td>
<td>0.36</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) (V-stem,V-loop) G20 G45</td>
<td>1.5</td>
<td>5.1</td>
<td>3.4</td>
<td>0.94</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) (V-stem,F-loop) G20 G45</td>
<td>1.8</td>
<td>5.5</td>
<td>3.1</td>
<td>0.86</td>
</tr>
<tr>
<td>tRNA^{Phe}(GAC) (F-stem,V-loop) G20 G45</td>
<td>16.5</td>
<td>2.1</td>
<td>0.13</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Additional mutants were constructed to clarify the contribution of nucleotides of the anticodon stem and loop to valine accepting activity. One tRNA^{Phe} variant, tRNA^{Phe}(V-stem, F-loop) G20 G45, has the anticodon stem of tRNA^{Val} (V-stem) but retains the anticodon loop of tRNA^{Phe} (F-loop) except for C36 (Figure 12). The other tRNA^{Phe} variant, tRNA^{Phe}(F-stem, V-loop) G20 G45, has the anticodon loop of tRNA^{Val} (V-loop) but retains the anticodon stem of tRNA^{Phe} (F-stem) (Figure 12). tRNA^{Phe}(V-stem, F-loop) G20 G45 is a very good substrate for ValRS with a relative $V_{max}/K_m$ of 0.86 compared to that of wild type tRNA^{Val} (Table 7). On the other hand, tRNA^{Phe}(F-stem, V-loop) G20 G45 is a relatively poor substrate (Table 7). These results indicate that determinant(s) for converting tRNA^{Phe}(GAC) G20 into an efficient substrate for valyl-tRNA synthetase are in the anticodon stem, and that G45 is needed for maximal aminoacylation activity.

**Role of 3'-'CCA of Aminoacyl-tRNA in Polypeptide Synthesis**

**Function of 3'-'CCA Variants of Valyl-tRNA^{Val} in Polypeptide Synthesis**

**Activity of valyl-tRNA^{Val} variants with substitution for 3'-'terminal adenine**

Our laboratory has reported that the universally conserved 3'-'CCA sequence in tRNA^{Val} is not essential for all tRNA functions (Liu and Horowitz, 1994). Substitution of the usual 3'-'terminal adenine (A) with either cytosine (C) or uracil (U) yields tRNA^{Val} variants that can still readily accept valine. Although tRNA^{Val} with a terminal guanine (G), can be fully aminoacylated, it is a much poorer substrate for ValRS (see Introduction). When the aminoacylated tRNA^{Val} variants were tested in a poly(U,G)-directed (Val, Phe) co-
polypeptide synthesis system (Ofengand et al., 1974), the tRNA\textsuperscript{Val} variant terminating in G is quite active, whereas the C and U variants are inactive (Figures 13 and 14). Aminoacylated 3'-CCA variants of valyl-tRNA\textsuperscript{Val} were used in these experiments to eliminate the effects of differences in aminoacylation efficiency among the tRNA\textsuperscript{Val} variants. With wild type valyl-tRNA\textsuperscript{Val}, 15 pmol of valine are incorporated into hot TCA precipitable material (\textit{in vitro synthesized polypeptide}) after 3 hours of incubation. This represents more than 16% of the input valyl-tRNA\textsuperscript{Val} (90 pmol). G76 valyl-tRNA\textsuperscript{Val} is also an active amino acid donor in polypeptide synthesis (Figure 13), however the rate of incorporation of valine into polypeptide is lower than that from the wild type tRNA. After 3 hours of incubation, about 5 pmol of the total 90 pmol of valine is incorporated. In both wild type and G76 valyl-tRNA\textsuperscript{Val}, the incorporation of valine into the polypeptide is almost linear over the three-hour time period. The U76 and C76 variants are inactive or very poor amino acid donors in polypeptide synthesis (Figure 13).

Activity of valyl-tRNA\textsuperscript{Val} variants with substitution for cytidines near the 3'-end

The activity of valyl-tRNA\textsuperscript{Val} variants with substitutions at positions 74 and 75 as valine donors in \textit{in vitro} poly(U\textsubscript{3},G)-directed (Val, Phe) co-polypeptide synthesis assays was also determined. The amounts of hot TCA precipitable radioactivity (polypeptide) after one hour of incubation were recorded for each mutant relative to the value obtained for wild type valyl-tRNA\textsuperscript{Val} (Figure 14). The amount of radioactive valine incorporated into polypeptide from the mutant tRNAs is between 30 to 60% of that from wild type valyl-tRNA\textsuperscript{Val}. This is also true for the double mutant, U74U75 (-UUA), even though it is a poor substrate for
Figure 13. Poly(U₃,G)-directed (Val,Phe) copolypeptide synthesis. The time course of [³H]-valine incorporation into polypeptide from wild type tRNA^Val (-CCA; closed circles), G76 variant (-CCG; open circles), U76 variant (-CCU; closed triangles), and C76 variant (-CCC; open triangles).
Figure 14. *In vitro* poly(U,G)-directed (Val,Phe) copolypeptide synthesis of valyl-tRNA$^{\text{Val}}$ variant in the 3'-end.
aminoacylation. These results indicate that mutations at position 74 and 75 do not severely affect the amino acid donor activity of valyl-tRNA\textsuperscript{Val}.

**Interaction of Elongation Factor Tu:GTP and Aminoacyl-tRNA**

The co-polypeptide synthesis assay determines the ability of different tRNA\textsuperscript{Val} variants to successfully complete several rounds of the elongation cycle to produce the acid precipitable (Val, Phe) co-polypeptide. Experiments were designed to identify the step or steps during the elongation cycle at which the pyrimidine substituted U76 and C76 tRNA\textsuperscript{Val} are not functional. The elongation cycle can be broken down into four discrete steps: (1) ternary complex formation, (2) binding of aminoacyl-tRNA at the A-site, (3) peptide bond formation at the peptidyltransferase center, and (4) translocation step/P-site binding (see Introduction). Identifying the step(s) at which the function of tRNA\textsuperscript{Val} mutants is impaired will contribute to an understanding of interactions between tRNA and components of the protein synthesis machinery, such as elongation factor Tu (EF-Tu), ribosomal RNA/protein, etc.

**Ternary complex formation with 3'-terminal variants of valyl-tRNA\textsuperscript{Val}**

The ability of aminoacylated native tRNA\textsuperscript{Val} (containing naturally occurring nucleotide modifications), wild type transcribed tRNA\textsuperscript{Val}, and each of the position-76 variants (G, C, and U) to form a ternary complex with EF-Tu and GTP was determined by a hydrolysis protection assay. The aminoacyl-tRNA linkage is unstable and is readily hydrolyzed; the rate of hydrolysis of valyl-tRNA\textsuperscript{Val} is dependent on pH. As the pH increases, the rate of spontaneous hydrolysis of the aminoacyl-ester linkage increases (results not shown). Valyl-tRNA\textsuperscript{Val} has the most stable aminoacyl-ester linkage of all aminoacyl-tRNAs,
and all mutant valyl-tRNA\textsuperscript{Val} variants are hydrolyzed at the same rate. Formation of a ternary complex with EF-Tu:GTP protects the aminoacyl ester bond from hydrolysis. As a result of preliminary tests (results not shown), the hydrolysis protection experiments with tRNA\textsuperscript{Val} were carried out at pH 8.0 and 37°C.

Figure 15 shows that in the absence of the elongation factor (curve labeled BSA), hydrolysis of the ester linkage follows a first order decay rate; after 3 hours, about 10% of the original valyl-tRNA\textsuperscript{Val} remains unhydrolyzed. In the presence of a 10-fold molar excess of EF-Tu:GTP, the elongation factor protects the aminoacyl bond of the wild-type and the G76 variant of valyl-tRNA\textsuperscript{Val} from hydrolysis (Figure 15); in both cases, nearly 95% of valyl-tRNA\textsuperscript{Val} remains intact. The ester linkage of the U76 mutant of valyl-tRNA\textsuperscript{Val} is also protected from hydrolysis by EF-Tu:GTP, but to a lesser degree; about 80% of U76 valyl-tRNA\textsuperscript{Val} remains unhydrolyzed after 3 hours of incubation (Figure 15). In the C76 mutant of valyl-tRNA\textsuperscript{Val}, the aminoacyl bond is, however, only minimally protected from hydrolysis by EF-Tu:GTP; the rate of hydrolysis approaches that of valyl-tRNA\textsuperscript{Val} in the absence of EF-Tu:GTP (Figure 15). Since in the absence of EF-Tu:GTP the rate of spontaneous hydrolysis of the ester bond is the same for all valyl-tRNA\textsuperscript{Val} variants regardless of the nature of nucleotide at the 3′-terminal position (Figure 15; curve labeled BSA), differences in the rates of hydrolysis in the presence of EF-Tu:GTP are indicative of EF-Tu:GTP’s affinity for a particular valyl-tRNA\textsuperscript{Val} variant. The hydrolysis protection results (Figure 15) clearly indicate that the mutants with 3′-terminal pyrimidines, particularly the C76 variant, are discriminated against in the formation of a ternary complex. EF-Tu:GTP evidently has a greater affinity for valyl-tRNA\textsuperscript{Val} terminating in purines.
Figure 15. Ternary complex formation between EF-Tu•GTP and *E. coli* valyl-tRNA<sup>Val</sup> at pH 8.0. The rate of hydrolysis of valylated wild type tRNA<sup>Val</sup>, 0.3 μM (A76; closed circles) and variants (0.3 μM) with nucleotide substitutions at their 3' termini: G76 (open circles), U76 (closed triangles), and C76 (open triangles) was measured in the presence of a 10-fold molar excess of EF-Tu•GTP. The rate of hydrolysis of valyl-tRNA<sup>Val</sup> in the presence of bovine serum albumin (BSA) is shown for comparison (closed squares).
Ternary complex formation with valyl-trNA$^{\text{Val}}$ variants having substitution for the cytidines near the 3′-end

Similar hydrolysis protection assays were carried out to monitor the interaction with EF-Tu:GTP of valyl-trNA$^{\text{Val}}$ variants with substitutions at position 74 and/or 75 of the universally conserved 3′-CCA end. For mutants at position 74, the results of the hydrolysis protection assay are shown on Figure 16. EF-Tu:GTP protects the aminoacyl ester bond of all position 74 mutants of valyl-trNA$^{\text{Val}}$ against hydrolysis as well as that of wild type valyl-trNA$^{\text{Val}}$. In all cases, a 10-fold molar excess of EF-Tu:GTP completely protects the aminoacyl linkage from hydrolysis at pH 8.0 for a 90-minute incubation at 37°C.

For tRNA$^{\text{Val}}$ mutants at position 75, the results of the hydrolysis protection assay are shown on Figure 17. The results were similar to those for mutants at position 74. In the presence of 10-fold molar excess of EF-Tu:GTP, all mutants at position 75 show complete protection of their aminoacyl ester bond at pH 8.0 over a 90-minute incubation at 37°C.

The hydrolysis protection of the U74U75 double mutant (3′-end sequence -UUA) by EF-Tu:GTP was also monitored. This variant is completely protected from spontaneous hydrolysis in the presence of 10-fold molar excess of EF-Tu:GTP (Figure 16).

Dissociation constants of ternary complexes

The hydrolysis protection assay provides a qualitative measure of the ability of aminoacyl-trNA to interact with EF-Tu:GTP. To obtain a more quantitative assessment, dissociation constants of the ternary complexes were determined using a ribonuclease protection assay (Louie and Jurnak, 1985; see Methods). The dissociation constant ($K_d$) of the ternary complex with native valyl-trNA$^{\text{Val}}$ was determined to be 8 nM (Table 8), this
Figure 16. Ternary complex formation between EF-Tu:GTP and *E. coli* valyl-tRNA\(^{\text{Val}}\) at pH 8.0. The rate of hydrolysis of valylated wild type tRNA\(^{\text{Val}}\), 0.3 µM (C74; closed circles) and variants (0.3 µM) with nucleotide substitutions at position 74: A74 (open circles), G74 (closed triangles), and U74 (open triangles) was measured in the presence of a 10-fold molar excess of EF-Tu:GTP. The rate of hydrolysis of a double mutant (U74U75; open squares) was also measured. The rate of hydrolysis of valyl-tRNA\(^{\text{Val}}\) in the presence of bovine serum albumin (BSA) is shown for comparison (closed squares).
Figure 17. Ternary complex formation between EF-Tu:GTP and *E. coli* valyl-tRNA\(^{\text{Val}}\) at pH 8.0. The rate of hydrolysis of valylated wild type tRNA\(^{\text{Val}}\), 0.3 μM (C75; closed circles) and variants (0.3 μM) with nucleotide substitutions at penultimate position: A75 (open circles), G75 (closed triangles), and U75 (open triangles) was measured in the presence of a 10-fold molar excess of EF-Tu:GTP. The rate of hydrolysis of valyl-tRNA\(^{\text{Val}}\) in the presence of bovine serum albumin (BSA) is shown for comparison (closed squares).
Table 8. Dissociation constants of ternary complexes with 3'-CCA variants of valyl-tRNA$^{\text{Val}}$
variants

<table>
<thead>
<tr>
<th>Valyl-tRNA$^{\text{Val}}$ Variants</th>
<th>$K_d$ (nM)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native tRNA$^{\text{Val}}$</td>
<td>8 ± 2.6</td>
<td>0.36</td>
</tr>
<tr>
<td>Wild type (A76)</td>
<td>22 ± 3.0</td>
<td>(1.0)</td>
</tr>
<tr>
<td>A76 → G76</td>
<td>47 ± 4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>A76 → U76</td>
<td>351 ± 45</td>
<td>16</td>
</tr>
<tr>
<td>A76 → C76</td>
<td>&gt; 1 μM</td>
<td>&gt; 45</td>
</tr>
<tr>
<td>C74 → A74</td>
<td>39 ± 1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>C74 → G74</td>
<td>25 ± 2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>C74 → U74</td>
<td>47 ± 2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>C75 → A75</td>
<td>35 ± 2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>C75 → G75</td>
<td>19 ± 3.2</td>
<td>0.9</td>
</tr>
<tr>
<td>C75 → U75</td>
<td>26 ± 2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>C74 C75 → U74 U75</td>
<td>27 ± 3.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>
agrees well with the previously reported value of 7.1 nM (Louie and Jurnak, 1985). The $K_d$ of the ternary complex with *in vitro* transcribed wild-type valyl-tRNA$_{Val}$ is somewhat higher, 22 nM. Nucleotide modifications in tRNA evidently increase the affinity of EF-Tu for valyl-tRNA$_{Val}$. $K_d$ values for complexes with G76 and U76 valyl-tRNA$_{Val}$ are 47 and 351 nM respectively, while that for the C76 mutant is considerably higher and is estimated to be in the micromolar range (Table 8).

The $K_d$ values for the ternary complexes of position 74 and 75 mutants of tRNA$_{Val}$, determined by the ribonuclease protection assay, are listed in Table 8. $K_d$ values are all within 2.1-fold that of the ternary complex with wild type valyl-tRNA$_{Val}$.

Ternary complex formation with 3'-terminal variants of alanyl-tRNA$_{Ala}$ and phenylalanyl-tRNA$_{Phe}$

To examine whether base-specific recognition of the 3'-terminus by EF-Tu:GTP can be generalized to other aminoacyl-tRNAs, ternary complex formation with 3'-terminal variants of *E. coli* tRNA$_{Ala}$ and tRNA$_{Phe}$ was also studied. The wild-type transcript of tRNA$_{Ala}$ and each of the position-76 variants (G, C, and U) can be fully aminoacylated with alanine at high concentrations of purified alanyl-tRNA synthetase (AlaRS) (Liu, 1995). Hydrolysis protection assays performed with alanyl-tRNA$_{Ala}$ variants (Figure 18), gave results similar to those found with valyl-tRNA$_{Val}$ variants (Figure 15). As in the hydrolysis protection experiments with valyl-tRNA$_{Val}$, those with alanyl-tRNA$_{Ala}$ were carried out at pH 8.0. Wild type alanyl-tRNA$_{Ala}$ and the G76 mutant readily form a ternary complex; the U76 mutant interacts with EF-Tu:GTP also, but to a lesser extent. Cytosine at the 3'-terminus greatly reduces formation of the ternary complex with EF-Tu:GTP. In the absence of
Figure 18. Ternary complex formation between EF-Tu:GTP and *E. coli* alanyl-tRNA<sup>Ala</sup> at pH 8.0. The rate of hydrolysis of alanylated wild type tRNA<sup>Ala</sup>, 0.3 μM (A76; open circles) and variants (0.3 μM) with nucleotide substitutions at their 3' termini: G76 (closed circles), U76 (closed triangles), and C76 (open triangles) was measured in the presence of a 10-fold molar excess of EF-Tu:GTP. The rate of hydrolysis of alanyl-tRNA<sup>Ala</sup> in the presence of bovine serum albumin (BSA) is shown for comparison (closed squares).
elongation factor (curve labeled BSA in Figure 18), the hydrolysis of all alanyl-tRNA$^{\text{Ala}}$ follows a first order decay rate. The ester linkage of alanyl-tRNA$^{\text{Ala}}$ is not as stable as that of valyl-tRNA$^{\text{Val}}$ at pH 8.0, and the degree of protection afforded by interaction with EF-Tu:GTP is somewhat lower than with valyl-tRNA$^{\text{Val}}$.

In experiments using partially purified phenylalanyl-tRNA synthetase (see Methods), it was found that tRNA$^{\text{Phe}}$ variants with base substitutions for the 3′-terminal A76 can be aminoacylated. The time course of aminoacylation of wild type tRNA$^{\text{Phe}}$ and 3′-terminal variants (C, G, and U) are shown on Figure 19. tRNA$^{\text{Phe}}$ C76 is readily aminoacylated whereas the G76 and U76 variants are poor substrates for aminoacylation. At high synthetase concentrations, the wild type and C76 variant of *E. coli* tRNA$^{\text{Phe}}$ could be fully aminoacylated, while the G76 and U76 variants of *E. coli* tRNA$^{\text{Phe}}$ were only 25% aminoacylated (results not shown).

Hydrolysis protection assays were performed at pH 8.0 with the phenylalanyl-tRNA$^{\text{Phe}}$ variants, and the results are similar to those observed with valyl-tRNA$^{\text{Val}}$ and alanyl-tRNA$^{\text{Ala}}$ variants (Figure 20). Wild-type phenylalanyl-tRNA$^{\text{Phe}}$ and the G76 mutant readily form ternary complexes; the U76 mutant of phenylalanyl-tRNA$^{\text{Phe}}$ is also protected by EF-Tu:GTP. This high level of protection is different from that of the U76 variants of tRNA$^{\text{Val}}$ and tRNA$^{\text{Ala}}$ (Figure 15 and 18). Again C at the 3′-terminus greatly reduces formation of the ternary complex with EF-Tu:GTP. In conclusion, the base-specific recognition of the 3′-terminal base is a general mechanism for recognition of aminoacyl-tRNAs by elongation factor Tu.
Figure 19. Time course of aminoacylation with phenylalanine of wild type *E. coli* tRNA^{Phe} (closed circles), tRNA^{Phe} C76 (open circles), tRNA^{Phe} G76 (closed triangles), and tRNA^{Phe} U76 (open triangles). RNA and phenylalanyl-tRNA synthetase concentrations are 2.6 μM and 0.04 μg/μl, respectively.
Figure 20. Ternary complex formation between EF-Tu:GTP and *E. coli* phenylalanyl-tRNA\(^\text{Phe}\) pH 8.0. The rate of hydrolysis of phenylalanylated wild type tRNA\(^\text{Phe}\), 0.3 \(\mu\)M (A76; closed circles) and variants (0.3 \(\mu\)M) with nucleotide substitutions at their 3' termini: G76 (open circles), U76 (closed triangles), and C76 (open triangles) was measured in the presence of a 10-fold molar excess of EF-Tu:GTP. The rate of hydrolysis of phenylalanyl-tRNA\(^\text{Phe}\) in the presence of bovine serum albumin (BSA) is shown for comparison (closed squares).
DISCUSSION

Identity Elements in E. coli tRNA^{Val}

To understand the molecular basis for the recognition of E. coli tRNA^{Val} by ValRS, steady-state kinetic studies of the aminoacylation of in vitro transcribed tRNA^{Val} variants were conducted to determine the effects of each mutation on activity. The importance of anticodon positions A35 and C36 as major recognition determinants of tRNA^{Val} was demonstrated by making nucleotide substitutions. Mutations introduced into these two positions severely reduced the aminoacylation activity of tRNA^{Val} variants (Schulman and Pelka, 1988; Tamura et al., 1991; Chu et al., 1992). The identity swap experiments with yeast tRNA^{Phe}, E. coli tRNA^{Phe}, and tRNA^{Ala} (Liu, 1995) further demonstrated and confirmed the importance of positions A35 and C36 of the anticodon in the recognition of E. coli tRNA^{Val} by ValRS. Introduction of C36 into E. coli and yeast tRNA^{Phe} and A35 into E. coli tRNA^{Ala} dramatically increased the aminoacylation efficiency of these tRNAs with valine. In addition, the discriminator base A73 (Liu, 1995; Tamura et al., 1991) and position G20 (Liu, 1995) in the D-loop were identified as recognition nucleotides of tRNA^{Val}. In the present study, kinetic analysis of tRNA^{Val} variants and identity swap experiments with E. coli tRNA^{Phe} identified G45 in tRNA^{Val} as a minor recognition nucleotide (Table 7). Our present recognition model for tRNA^{Val}-ValRS interaction specifies a set of five recognition nucleotides in tRNA^{Val}, these include A35, C36, A73, G20, and G45. In addition, a wobble base pair in the middle of the acceptor stem was found to affect the aminoacylation efficiency of tRNA^{Val} by ValRS (Table 1 and 5). The efficient recognition of tRNA^{Val} by ValRS requires the acceptor stem to be in a normal A-form helix. This conclusion is based on
results of kinetic studies of tRNA$^{Val}$ variants, identity swap experiments of yeast tRNA$^{Phe}$ and E. coli tRNA$^{Ala}$ (Liu, 1995), and $^{19}$F NMR experiments with 5-fluorouracil-substituted tRNA$^{Val}$ (Liu et al., 1997).

**Role of Acceptor Stem of tRNA$^{Val}$ in the Recognition by ValRS**

Even though many tRNAs have major recognition nucleotides in their acceptor stem helix, especially in the first three base pairs (see Introduction), kinetic studies of acceptor stem variants of tRNA$^{Val}$ did not reveal any recognition nucleotides in the acceptor stem helix. In general, aminoacylation activity of tRNA$^{Val}$ is not significantly affected by Watson-Crick base pair substitutions at different positions of the acceptor stem helix. These results differ from those of Tamura et al. (1991) (see Result) who reported that changing the U4:A69 base pair to A4:U69 decreases aminoacylation activity 5.3-fold and replacing G3:C70 with C3:G70 lowers activity 2.3-fold. This disagreement is not due to differences in experimental conditions: Tamura and coworkers used 19.2 μM valine (sub-optimal) in their aminoacylation reaction, and we used 100 μM (twice $K_m$). If mutations in a tRNA affect ValRS’s affinity for the amino acid in the aminoacylation reaction, as in the case of tRNA$^{Gln}$ (Ibba et al., 1996), then it is possible that the decrease in activity of the acceptor stem variants, observed by Tamura et al. (1991), is due to determinants for valine affinity in tRNA$^{Val}$. However, wild-type and acceptor stem variants of tRNA$^{Val}$ have similar $K_m$ for valine in the aminoacylation reaction (Table 2). Furthermore, aminoacylation kinetic studies of C3:G70 and A4:U69 variants under condition of sub-optimal valine concentration (10 μM) showed near wild-type activities (Table 3). These results indicate that Watson-Crick base pair mutations in the acceptor stem of tRNA$^{Val}$ do not affect the valine affinity of ValRS for
aminoacylation, and ValRS does not appear to recognize any specific base or base pair in the acceptor stem.

Introduction of G:U base pairs into the acceptor stem helix did not significantly affect aminoacylation activity except when the G:U base pair is located at position 4:69 or 3:70 to a lesser extent. tRNA_{Val} variants at position 4:69 can be divided into two groups: one with good aminoacylation activity and one with relatively poor activity (Table 9). The base pair U4:A69 in the center of the acceptor stem of tRNA_{Val} can be substituted by other Watson-Crick base pairs such as C4:G69, and A4:U69 or even the non-canonical base pair U4:C69 with only a relatively small decrease in the specificity constant (V_{max}/K_m) for aminoacylation. In general, tRNA_{Val} variants, having a base pair at positions 4:69 that maintains a normal A-form acceptor stem helix, have good aminoacylation activity; these variants have Watson-Crick base pairs or an U:C base pair at position 4:69. The U:C pair in a RNA double helix is believed to maintain a regular A-form helical structure as shown by the crystal structure of a 12 base-pair RNA duplex (Holbrook et al., 1991). The slightly lower activity observed with G4:C69 in the acceptor stem helix could be the result of increased rigidity of the acceptor stem helix rather than the absence of specific recognition at base pair 4:69. An energy calculation of intramolecular interactions between the bases in a double-stranded helix (DeVoe and Tinoco, 1962) showed that the largest contribution to helical stability came from vertical stacking of bases. Studies of oligoribonucleotides had shown that helical stability is strongly dependent on sequence (Borer et al., 1974). In particular, G:C over G:C is more stable than either G:C over C:G or C:G over G:C.
Table 9. Kinetic parameters for aminoacylation of tRNA^{val} variants at position 4:69 in the acceptor stem: A-Form Helix vs. Distorted Helix

<table>
<thead>
<tr>
<th>tRNA^{val} Variants</th>
<th>K_m (µM)</th>
<th>V_max (µmol/min/mg)</th>
<th>V_max/K_m</th>
<th>Relative (V_max/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A-Form Helix</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>1.4</td>
<td>5.0</td>
<td>3.6</td>
<td>(1.0)</td>
</tr>
<tr>
<td>U4:A69 → A4:U69</td>
<td>1.6</td>
<td>4.9</td>
<td>3.1</td>
<td>0.86</td>
</tr>
<tr>
<td>U4:A69 → C4:G69</td>
<td>1.4</td>
<td>4.3</td>
<td>3.0</td>
<td>0.83</td>
</tr>
<tr>
<td>U4:A69 → G4:C69</td>
<td>6.2</td>
<td>6.8</td>
<td>1.1</td>
<td>0.31</td>
</tr>
<tr>
<td>U4:A69 → U4*C69</td>
<td>2.9</td>
<td>5.8</td>
<td>2.0</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Distorted Helix</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U4:A69 → G4:U69</td>
<td>20.0</td>
<td>1.9</td>
<td>0.095</td>
<td>0.026</td>
</tr>
<tr>
<td>U4:A69 → U4:G69</td>
<td>10.4</td>
<td>6.2</td>
<td>0.60</td>
<td>0.17</td>
</tr>
<tr>
<td>U4:A69 → A4*G69</td>
<td>8.9</td>
<td>6.1</td>
<td>0.69</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*a,b Results from Liu (Liu, 1995) and Vahid Feiz shown here for comparison.
Therefore, the presence of five consecutive guanines on one strand and cytosines on the other in G4:C69 tRNA\textsuperscript{Val} variant (Figure 2) have the potential of having a very rigid acceptor stem helix.

On the other hand, tRNA\textsuperscript{Val} variants with poor activities have G:U base pairs or an A:G base pair at position 4:69 that can cause distortion of the normal A-form helical structure (Table 9). Substitution by the wobble base pair U4:G69 or G4:U69 results in a large decrease in specificity constant (Table 9). \textsuperscript{19}F NMR experiments of tRNA\textsuperscript{Val} variants with G:U base pairs at positions 2:71, 3:70 and a FU:G base pair at position 4:69 in the acceptor stem showed a gradual downfield shift of the resonance corresponding to FU4 toward a region of \textsuperscript{19}F NMR spectrum that corresponds to FU residues in single-stranded regions of tRNA\textsuperscript{Val} (Liu, 1995; Chu and Horowitz, unpublished). This is strong evidence for changes in the local structure of the acceptor stem helix around base pair 4:69 when a G:U base pair is present at or in close proximity to this location. A strong correlation between the chemical shift of resonance FU4 in the \textsuperscript{19}F NMR spectrum of different tRNA variants and their relative aminoacylation activity was also observed: as the degree of downfield shift of resonance FU4 in a tRNA variant increases, the aminoacylation activity of that tRNA variant decreases (Liu, 1995).

The local distortion of the acceptor stem helix in the presence of a G:U base pair is most likely due to differences in base stacking with the adjacent base pairs. G:U base pairs are a very common structural feature of RNA molecules. This structural feature is clearly evident in several crystallographic and NMR structures containing G:U base pairs in helical stems. On the basis of the yeast tRNA\textsuperscript{phe} crystal structure, Mizuno and Sundaralingam
(1978) predicted that base pairs to the 5' side of the U of a G:U pair and the 3' side of the G will stack favorably with the G:U, while base pairs on the opposite side should stack very poorly on the G:U. Available structures of RNA support this hypothesis, and this preferential base stacking is believed to cause a local helical distortion. In the NMR structure of P1 helix from Group I self-splicing introns, Allain and Varani, (1995), concluded from their analysis of the conserved G:U wobble base pair in a double helical structure that G:U wobble base pairs cause a local distortion to the regular A-form helix. In the crystallographic structure of yeast tRNA<sup>?</sup> (Westhof <i>et al.</i>, 1985), there are two G:U base pairs in the helical regions. Both G:U base pairs are flanked by two G:C base pairs, as in the case for the mutant tRNA<sup>?</sup> which has a G4:U69 base pair in the acceptor stem. For both G:U base pairs in the acceptor and anticodon stem helices of yeast tRNA<sup>?</sup>, a preferential base stacking was observed as predicted by Mizuno and Sundaralingam. In a NMR analysis of Helix I of the 5S RNA of <i>E. coli</i>, the G:U base pair also showed strong base stacking interaction with the base on the 5' side of the U and the 3' side of the G, while base pairs on the opposite side stacked very poorly (White <i>et al.</i>, 1992).

The identity swap experiments with yeast tRNA<sup>F</sup> (Table 5) and <i>E. coli</i> tRNA<sup>A</sup> (Liu, 1995) agree well with the results of G:U base pair substitutions at position 4:69 and 3:70 in tRNA<sup>?</sup>. In order for these tRNAs to be efficient substrates for ValRS, the G:U base pairs at position 3:70 of tRNA<sup>A</sup> and 4:69 of yeast tRNA<sup>F</sup> needed to be substituted with Watson-Crick base pairs. The G:U base pair in the acceptor stem of tRNA<sup>A</sup> and yeast tRNA<sup>F</sup> acts as a negative determinant to prevent recognition by ValRS.
A NMR study of an 8-base pair RNA duplex concluded that the local geometry of A:G pair in a RNA helix resembles that of a G:U wobble base pair (SantaLucia and Turner, 1993). An A4:G69 base pair substitution at this location also caused a significant decrease in specificity constant (Table 9). A large increase in $K_m$ was responsible for the decreases in specificity constant in this mutant tRNA. From these experimental results, it can be concluded that the U4:A69 base pair in tRNA$^{Val}$ does not contain functional groups that are directly recognized by ValRS, but ValRS needs to have a normal A-form helical structure at this position in the acceptor stem. The acceptor stem of tRNA$^{Val}$ may need to be in an A-form helix for proper positioning of the 3'-CCA into the active site of ValRS.

Even though the results of our kinetic studies do not indicate base-specific recognition of the acceptor stem of tRNA$^{Val}$ by ValRS, other results indicate that ValRS makes contact with the acceptor stem of tRNA$^{Val}$ during aminoacylation. RNase V1 footprinting experiments (Derrick, 1991) indicate that ValRS protects the 5' phosphodiester bonds of positions 66 and 69 in the acceptor stem from cleavage. In $^{19}$F-NMR experiments, interaction of ValRS with 5-fluorouracil-substituted tRNA$^{Val}$ results in a loss of intensity of resonances corresponding to FU67, and a broadening and shifting of FU4 (Chu and Horowitz, 1991).

**Role of Tertiary Interactions of tRNA$^{Val}$ in the Recognition by ValRS**

Four of the nine tertiary interaction, first identified in yeast tRNA$^{Phe}$, have conserved sequences in all non-mitochondrial tRNAs from prokaryotic to eukaryotic organisms (Table 10). Therefore, it is very unlikely that these nucleotides are used by the aminoacyl-tRNA synthetases to specifically recognize their respective cognate tRNAs. The remaining five
Table 10. Tertiary Interactions for *E. coli* elongator tRNAs

<table>
<thead>
<tr>
<th>Tertiary Interaction</th>
<th>% of tRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>G19-C56</td>
<td>100</td>
</tr>
<tr>
<td>G18-U55</td>
<td>100</td>
</tr>
<tr>
<td>U54-A58</td>
<td>100</td>
</tr>
<tr>
<td>U8-A14</td>
<td>100</td>
</tr>
<tr>
<td><strong>15-48</strong></td>
<td></td>
</tr>
<tr>
<td>G15-C48</td>
<td>85</td>
</tr>
<tr>
<td>A15-U48</td>
<td>10</td>
</tr>
<tr>
<td>G15-G48</td>
<td>5</td>
</tr>
<tr>
<td><strong>13-22-46</strong></td>
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</tr>
<tr>
<td>C13-G22-G46</td>
<td>70</td>
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<tr>
<td>others</td>
<td>30</td>
</tr>
<tr>
<td><strong>9-23-12</strong></td>
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</tr>
<tr>
<td>A9-A23-U12</td>
<td>65</td>
</tr>
<tr>
<td>C9-G23-C12</td>
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</tr>
<tr>
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</tr>
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<td>40</td>
</tr>
<tr>
<td>U45-G10-C25</td>
<td>20</td>
</tr>
<tr>
<td>G45-C10-G25</td>
<td>10</td>
</tr>
<tr>
<td>G45-G10-U25</td>
<td>10</td>
</tr>
<tr>
<td>others</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 10. (continued)

<table>
<thead>
<tr>
<th>Tertiary Interaction</th>
<th>% of tRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-44</td>
<td></td>
</tr>
<tr>
<td>A26-G44</td>
<td>30</td>
</tr>
<tr>
<td>G26-A44</td>
<td>20</td>
</tr>
<tr>
<td>A26-C44</td>
<td>20</td>
</tr>
<tr>
<td>A26-U44</td>
<td>10</td>
</tr>
<tr>
<td>others</td>
<td>20</td>
</tr>
</tbody>
</table>

tertiary interactions involve positions 15-48, 26-44, 13-22-46, 9-23-12, and 45-10-25; the last three involve triplet interactions. The frequency of occurrence of particular nucleotides in these five tertiary interaction are shown for all *E. coli* elongator tRNAs in Table 10. *E. coli* tRNA\(^{\text{Val}}\) happens to contain nine of the tertiary interactions most commonly found in *E. coli* elongator tRNAs. Tertiary nucleotides at positions 15-48, 13-22-46, and 9-23-12 of tRNA\(^{\text{Val}}\) are the same as those found at these positions in 85, 70, and 65%, respectively, of other *E. coli* elongator tRNAs. Hence, special attention is given to the remaining two tertiary interactions, 26-44 and 45-10-25, for their roles in the recognition process by valyl-tRNA synthetase.

A single base substitution of U45 for G45 in tRNA\(^{\text{Val}}\) results in a moderate decrease in aminoacylation efficiency (Table 1). The effect of an equivalent mutation on the aminoacylation efficiency of *E. coli* tRNA\(^{\text{Phe}}\) by *E. coli* PheRS was much more pronounced; a single base substitution of U45 to G45 resulted in a 7-fold reduction in specificity constant.
(Peterson and Uhlenbeck, 1992). The U45-G10-C25 tertiary base triplet was therefore identified as a recognition element of *E. coli* tRNA\(^\text{Phe}\) in the *E. coli* PheRS system. The importance of G45 in the aminoacylation of a tRNA by ValRS was not clearly evident until the results of the identity swap experiment with *E. coli* tRNA\(^\text{Phe}\) were examined. The aminoacylation activity with valine of *E. coli* tRNA\(^\text{Phe}\) variants was consistently increased by about 3-fold by the presence of G45 (as in tRNA\(^\text{Val}\)) in place of the U45 present in tRNA\(^\text{Phe}\) (Table 6 and 7). These results suggest that G45 is involved in the recognition of tRNA\(^\text{Val}\) by ValRS. U45 of *E. coli* tRNA\(^\text{Phe}\) is the only other recognition nucleotides identified in the variable loop of a tRNA with a short variable loop. The variable loops of *E. coli* tRNA\(^\text{Ser}\) and tRNA\(^\text{Tyr}\), both with long variable loops, have been implicated in the recognition process of their respective synthetases (Himeno *et al.*, 1990).

Single base substitutions of A26 to G26 or G44 to A44 result in moderate decreases in aminoacylation activity (Table 4). These results are in contrast to the same mutations of yeast tRNA\(^\text{Phe}\) that showed wild type activities in the yeast phenylalanyl-tRNA synthetase (PheRS) system (Sampson *et al.*, 1990) Aminoacylation kinetic studies of these yeast tRNA\(^\text{Phe}\) variants indicated that they all have lower \(K_m\) values which result in higher specificity constants for these variants than for wild type tRNA\(^\text{Phe}\). Perhaps the mutations at this tertiary base pair in the sequence context of yeast tRNA\(^\text{Phe}\) are less disruptive to the overall structure, or yeast phenylalanyl-tRNA synthetase is more tolerant of possible conformational changes caused by these mutations. A double-mutation in tRNA\(^\text{Val}\), A26-G44 \(\rightarrow\) G26-A44, that restores an A:G interaction increased the aminoacylation activity to the level of wild type tRNA\(^\text{Val}\) (Table 4). The A26-G44 tertiary interaction in *E. coli* tRNA\(^\text{Phe}\)
were identified as recognition nucleotides because a structurally conservative substitution of
G26-A44 caused a 14-fold decrease in activity (Peterson and Uhlenbeck, 1992). This E. coli
tRNA\textsuperscript{Phe} variant is believed to be structurally similar to its wild type counterpart based on its
normal lead cleavage rate (Peterson and Uhlenbeck, 1992). Lead cleavage in tRNA\textsuperscript{Phe} is very
sensitive to the integrity of the core region of a tRNA (Behlen \textit{et al.}, 1990). The present \textit{in
vitro} analyses showed that the 26-44 tertiary interaction is not critical for aminoacylation of
\textit{tRNA}\textsuperscript{Val} as long as the A-G/G-A tertiary interaction is maintained.

\textbf{Identity Swap Experiments}

Availability of three active chimeric substrates for ValRS: yeast tRNA\textsuperscript{Phe} (GAC) A4
(Table 5), \textit{E. coli} tRNA\textsuperscript{Phe} (GAC) (V-stem, F-loop) G20 G45 (Table 7), and \textit{E. coli}
tRNA\textsuperscript{Ala} (UAC) A3:U70/G3:C70 (Liu, 1995), makes it possible to eliminate a substantial
number of additional nucleotides as potential recognition sites for ValRS. For example, the
G5:C68 base pair in the acceptor stem of tRNA\textsuperscript{Val} is A5:U68 and C5:G68 in yeast tRNA\textsuperscript{Phe}
(GAC) A4 and \textit{E. coli} tRNA\textsuperscript{Ala} (UAC) A3:U70/G3:C70, respectively. Of the 76 nucleotides
in tRNA\textsuperscript{Val}, 23 can be eliminated from consideration because they are either conserved or
semi-conserved nucleotides in all \textit{E. coli} tRNAs. Of the remaining 53 nucleotides, 7 have
been changed to at least two other nucleotides and an additional 13 to one other nucleotide
without significantly altering the aminoacylation kinetics. Of the remaining 33 nucleotides, 4
nucleotides at base pairs 4:69 and 29:41 are not specifically recognized by ValRS; rather the
wobble base pair at position 4:69 and the C:G base pair at position 29:41 induce structural
features that are unfavorable for efficient aminoacylation. Of the remaining 29 nucleotides,
identity swap experiment with yeast tRNA\textsuperscript{Phe} helped to exclude 12. The efficient valine-
accepting chimeric yeast tRNA\textsuperscript{Phe}, tRNA\textsuperscript{Phe}(GAC) A4 (Table 5), has eight nucleotides in the T-stem, position 16, base pairs 5:68 and 31:59 that are different from those in tRNA\textsuperscript{Val}. Seven of the eight nucleotides in the T-stem of tRNA\textsuperscript{Val} have never been individually tested as synthetase recognition nucleotides. Finally, five of the remaining 17 nucleotides have been identified as recognition nucleotides and the remainder (mainly in the D-Stem and the top two base pairs in the anticodon stem) have not yet been tested. Thus, a limited number of additional nucleotides for ValRS recognition may still be possible.

The ability to switch the identity of yeast tRNA\textsuperscript{Phe}(GAA) to a valine acceptor by making two nucleotide substitutions, A36 to C36 and G4 to A4, suggests that the overall structure of tRNA\textsuperscript{Val} is very similar to that of yeast tRNA\textsuperscript{Phe}. This similarity allows us to visualize the recognition nucleotides of tRNA\textsuperscript{Val} in the scaffold of the well-established crystal structure of yeast tRNA\textsuperscript{Phe} (Figure 21). The recognition nucleotides reside in four regions of the tRNA tertiary structure: the variable pocket (G20), the central core (G45), the anticodon loop (A35 and C36), and the acceptor end (A73). All four regions are also sites at which other tRNA synthetases interact with tRNA as shown by both \textit{in vivo} and \textit{in vitro} experiments (Schulman, 1991; Pallanck \textit{et al.}, 1995).

Although the identity swap experiments with yeast tRNA\textsuperscript{Phe} and \textit{E. coli} tRNA\textsuperscript{Ala} (Liu, 1995) proved successful, the identity swap experiment with \textit{E. coli} tRNA\textsuperscript{Phe} was not completely satisfying. \textit{E. coli} tRNA\textsuperscript{Phe} was converted to an efficient substrate for ValRS only after wholesale substitution of the entire anticodon stem, in addition to converting A36 to C36, U20 to G20, and U45 to G45 (Table 7). It is clear from aminoacylation kinetic
Figure 21. Synthetase recognition nucleotides of *E. coli* tRNA$^{Val}$ in the structural backbone of yeast tRNA$^{Phe}$. The tRNA backbone is depicted in black, and the recognition nucleotides are shown in gray. The structure was generated by the program RasMol (R. Sayle).
studies of tRNA\textsuperscript{Val} variants that several mutations at base pairs 29:41 and 30:40 yield active substrates for ValRS (Liu, 1995). The anticodon stems of active chimeric yeast tRNA\textsuperscript{Phe}(GAC) A4 and \textit{E. coli} tRNA\textsuperscript{Ala}(UAC)A3:U70/G3:C70 (Liu, 1995) differ from tRNA\textsuperscript{Val} in three base pairs and one base pair, respectively. Wrede \textit{et al}. (1979) found that the conformation of the anticodon loop of initiator tRNAs can be influence by the sequence of the anticodon stem. Therefore, base substitutions in the anticodon stem of \textit{E. coli} tRNA\textsuperscript{Phe} may be needed for the anticodon loop to have the correct conformation needed for productive recognition by ValRS. Giege \textit{et al}. (1993) pointed out that nucleotides that reside close to one another in the tertiary structure may define a unique structural feature of that region of the tRNA molecule. It is possible for the same mutations to have different effects in the sequence context of different tRNAs. The identity swap experiments with \textit{E. coli} tRNA\textsuperscript{Phe} were not unique. In fact, Peterson and Uhlenbeck (1992) reported that the most active chimeric substrates of yeast tRNA\textsuperscript{Phe} and \textit{E. coli} tRNA\textsuperscript{Met} for \textit{E. coli} phenylalanyl-tRNA synthetase have about 30% and 2%, respectively, of wild-type \textit{E. coli} tRNA\textsuperscript{Phe} activity. Schulman and Pelka (1988) found that tRNA\textsuperscript{Met} with a valine anticodon has only 10% of the wild-type tRNA\textsuperscript{Val} activity.

The studies on \textit{E. coli} tRNA\textsuperscript{Val} variants and the identity swap experiments have shown the overwhelming importance of the anticodon bases A35 and C36 as synthetase recognition nucleotides. The discriminator base A73 and G20 in the variable pocket had also been shown to be recognized by ValRS in a specific manner. G45 of tRNA\textsuperscript{Val} is identified as a recognition nucleotide mainly through identity swap experiments with \textit{E. coli} tRNA\textsuperscript{Phe}. Our study also suggests that the A-form conformation of the acceptor stem, especially around
base pair 4:69, is recognize by ValRS. The present view of the identity elements of tRNA\textsuperscript{Val} includes five nucleotides: A35, C36, A73, G20 and G45, plus an A-form helical conformation of the acceptor stem.

**Role of 3′-CCA of Aminoacyl-tRNA in Polypeptide Synthesis**

**Function of 3′-CCA Variants of Val\textsuperscript{Val}-tRNA\textsuperscript{Val} in Polypeptide Synthesis**

The universal conservation of 3′-CCA sequence suggests that it is critical for the function of tRNA in polypeptide synthesis. However, Liu (1995) has found that many tRNA\textsuperscript{Val} variants with modifications at the universally conserved 3′-CCA are substrates for ValRS in aminoacylation. The C76 and U76 tRNA\textsuperscript{Val} variants are highly efficient substrates for ValRS, while G76 variant is a very poor substrate (Liu and Horowitz, 1994). The ability of tRNA\textsuperscript{Val} with altered 3′-CCA sequence to function in polypeptide formation in vitro was tested in a poly(U3,G)-directed (Val, Phe) co-polypeptide synthesis system (Liu, 1995).

Amino acid donor activity of tRNA\textsuperscript{Val} variants were tested using aminoacylated tRNA\textsuperscript{Val} in order to eliminate differences in aminoacylation efficiency of different tRNA\textsuperscript{Val} variants. Previous *in vitro* polypeptide synthesis experiments (Liu, 1995) used the heterologous yeast tRNA\textsuperscript{Phe} as the phenylalanine donor in the copolypeptide synthesis system. The present experiments utilized *E. coli* tRNA\textsuperscript{Phe} as the phenylalanine donor and resulted in a more efficient synthesis of polypeptide. In agreement with the results of Liu (1995), the present study shows that the 3′-CCA variants with terminal purines are active valine donors in polypeptide synthesis whereas C76 and U76 tRNA\textsuperscript{Val} variants are poor valine donors (Figure 14) (see also Liu and Horowitz, 1994). Even though C76 and U76 tRNA\textsuperscript{Val} variants are
highly active in aminoacylation, they are inactive valine donors for polypeptide synthesis (Figure 13). On the other hand, the G76 tRNA$^{Val}$ variant, which is a very poor substrate for aminoacylation, is an active valine donor in polypeptide synthesis, having 40% of the valine donor activity of wild-type tRNA$^{Val}$ (Figure 13 and 14).

**Interaction of Aminoacyl-tRNA and EF-Tu**

To identify the step or steps in the polypeptide chain elongation cycle at which the tRNA$^{Val}$ mutants terminating in 3'-pyrimidines, U76 and C76, fail to function, the interaction of aminoacylated mutant tRNAs with EF-Tu:GTP was examined by a hydrolysis protection assay in which interaction of aminoacyl-tRNA with EF-Tu:GTP protects the labile aminoacyl linkage from hydrolysis (see Methods).

**Specific recognition of 3'-terminal base of valyl-tRNA$^{Val}$ by EF-Tu:GTP**

The hydrolysis protection assay is a reliable qualitative measure of aminoacyl-tRNA/EF-Tu:GTP interaction. A similar hydrolysis protection assay was used to show that a 10 base-pair helix linked to the 3'-NCCA sequence is the minimal helical length for efficient interaction with elongation factor Tu (Rudinger et al., 1994). The results of the hydrolysis protection experiments (Figure 15) clearly indicate that the tRNA$^{Val}$ mutant with a 3'-terminal cytidine is discriminated against in the formation of a ternary complex. The rates of hydrolysis of C76 valyl-tRNA$^{Val}$ in the presence and absence of EF-Tu:GTP are almost indistinguishable (Figure 15). To a lesser degree, EF-Tu:GTP also shows a decreased affinity for the U76 mutant of valyl-tRNA$^{Val}$. On the other hand, both wild type (A76) and the G76 mutant of valyl-tRNA$^{Val}$ have high affinities for EF-Tu, as indicated by the complete protection of both valyl-tRNA$^{Val}$ from spontaneous hydrolysis in the presence of EF-Tu:GTP.
(Figure 15). Similar results were observed with 3'-terminal mutants of *E. coli* alanyl-tRNA$^{\text{Ala}}$ and phenylalanyl-tRNA$^{\text{Phe}}$ (Figures 18 and 20). Wild type (A76) and the G76 mutant of alanyl-tRNA$^{\text{Ala}}$ and phenylalanyl-tRNA$^{\text{Phe}}$ show high affinities for EF-Tu. The presence of 3’-terminal cytidine causes both aminoacyl-tRNAs to lose their affinity for EF-Tu. The U76 mutant of phenylalanyl-tRNA was protected unexpectedly well in the hydrolysis assay. It appears that the presence of pyrimidines, especially cytidine, in the 3’-terminal position of aminoacyl-tRNA inhibit the formation of the ternary complex.

The aminoacyl ester bond of valyl-tRNA$^{\text{Val}}$ has a half-life of about one hour at pH 8.0 (Figure 15). Binding of aminoacyl-tRNA to EF-Tu:GTP inhibits the rate of this spontaneous hydrolysis (Pingoud and Urbanke, 1980). The molecular details of this protection are not known (Rudinger *et al.*, 1994), and in the time range used for this assay (180 minutes) the intrinsic GTPase activity of EF-Tu converts a considerable amount of GTP to GDP (Chinali *et al.*, 1977). This reaction can affect the equilibrium for ternary complex formation. The data for the hydrolysis protection assays, should therefore, be considered only as a qualitative demonstration of the presence or the absence of the interaction between aminoacyl-tRNA and EF-Tu:GTP.

**K₄ discrimination of 3’-terminal base of valyl-tRNA$^{\text{Val}}$**

Quantitative determination of the dissociation constants of ternary complexes between EF-Tu:GTP and 3’-CCA variants of tRNA$^{\text{Val}}$ was carried out using a ribonuclease protection assay (Table 8). This method was first developed by Louie and Jurnak (1985) and was used to determine the dissociation constants of ternary complexes with the complete set of aminoacyl-tRNAs including at least one tRNA coding for each of the 20 amino acids. In
the present study, the dissociation constant \((K_d)\) of the ternary complex with native modified valyl-tRNA\(^{\text{val}}\) was determined to be 8 nM (Table 8), a value which agrees well with that of 7.1 nM reported by Louie and Jurnak, (1985). Valyl-tRNA\(^{\text{val}}\) forms the weakest complex of all noninitiator EF-Tu:GTP:aminoacyl-tRNA complexes. This may reflect the fact that the ester linkage of valyl-tRNA\(^{\text{val}}\) is the most stable among all aminoacyl-tRNAs. In vitro transcribed wild-type valyl-tRNA\(^{\text{val}}\) has a 2.75-fold higher \(K_d\), 22 nM (Table 8). Nazarenko et al. (1994) reported that the \(K_d\) for the ternary complex with unmodified yeast phenylalanyl-tRNA\(^{\text{phe}}\) is two-fold higher than that of the native counterpart. These results indicate that nucleotide modifications in tRNA increase the affinity of EF-Tu:GTP for aminoacyl-tRNA.

C76 valyl-tRNA\(^{\text{val}}\) variant interacts poorly with EF-Tu as indicated earlier by the hydrolysis protection assay. This poor interaction makes determination of the dissociation constant of the ternary complex with the C76 valyl-tRNA variant inaccurate, and the \(K_d\) was estimated to be in the micromolar range, which is at least 45-fold higher than the \(K_d\) for the ternary complex involving wild-type valyl-tRNA\(^{\text{val}}\) (Table 8). Even though U76 mutants of the various aminoacyl-tRNAs tested showed substantial affinity for EF-Tu:GTP, as indicated by their significant protection from spontaneous hydrolysis in the presence of EF-Tu:GTP (Figure 15, 18 and 20), the dissociation constant of the ternary complex with U76 valyl-tRNA\(^{\text{val}}\) is 16-fold higher, 351 nM, than the \(K_d\) of the ternary complex with wild-type valyl-tRNA\(^{\text{val}}\) (Table 8). This difference in \(K_d\) is significant and compares with similar values obtained with the initiator tRNA\(^{\text{ile}}\) (Louie and Jurnak, 1985). Initiator methionyl-tRNA\(^{\text{ile}}\) forms a weak complex with EF-Tu:GTP (Tanada et al., 1982). Its dissociation constant was
found to be 15.5-fold higher than that of the complex with native valyl-tRNA$^{\text{Val}}$ (Louie and Jurnak, 1985). This 15.5-fold increase in $K_d$ is sufficient to prevent initiator methionyl-tRNA$^{\text{Met}}$ from participating in the translation elongation cycle. The authors indicate that the weaker ternary complex formation with initiator methionyl-tRNA$^{\text{Met}}$ is due to a faster dissociation of the errant complex. This suggests that EF-Tu:GTP initially recognizes all aminoacyl-tRNAs almost equivalently, but that proper selection of the noninitiator aminoacyl-tRNAs occurs by the rapid rejection of the initiator tRNA species.

We now have a possible explanation for the observation that the C76 and U76 valyl-tRNA$^{\text{Val}}$ variants are inactive valine donors in \textit{in vitro} polypeptide synthesis. A pyrimidine at the 3'-terminal position of valyl-tRNA$^{\text{Val}}$ is discriminated against because elongation factor Tu specifically recognize a purine at this position. The inability of the C76 and U76 valyl-tRNA$^{\text{Val}}$ variants to form ternary complexes with EF-Tu:GTP excludes them from participating in the elongation process. This conclusion is based on the protection of the wild-type and G76 valyl-tRNA$^{\text{Val}}$ variants by EF-Tu against deacylation and the lack of protection of the C76 and U76 valyl-tRNA$^{\text{Val}}$ variants, and on the determination of dissociation constants for the interaction of various valyl-tRNA$^{\text{Val}}$ variants with EF-Tu:GTP. However, this study does not rule out the possibility that C76 and U76 valyl-tRNA$^{\text{Val}}$ are also inactive in other steps of the elongation cycle, such as the peptidyltransferase reaction and translocation. In addition, we found that the specific recognition of a purine at the 3'-terminal position of aminoacyl-tRNA is general because the hydrolysis protection assay with 3'-terminal variants of \textit{E. coli} alanyl-tRNA$^{\text{Ala}}$ and phenylalanyl-tRNA$^{\text{Phe}}$ show results similar to those with the 3'-terminal variants of valyl-tRNA$^{\text{Val}}$. 
What is the molecular basis for the specific recognition of a purine at the 3'-terminal position of aminoacyl-tRNA by EF-Tu? When comparing the four bases (adenine, guanine, cytosine, and uracil) in regard to the geometric location of functional groups and the potential hydrogen bonding donors and acceptors on each base, only the heterocyclic nitrogen at position 7 on adenine and guanine distinguishes the purines from the pyrimidines. The recently available crystal structure of the ternary complex between yeast phenylalanyl-tRNA^{Phe} and EF-Tu (see Introduction) indicates that contacts between EF-Tu and phenylalanyl-tRNA^{Phe} are very limited. The 3'-terminal adenine of phenylalanyl-tRNA^{Phe}, identified from its electron density, is located in a hydrophobic pocket flanked by several hydrophobic amino acid residues of elongation factor Tu (see Introduction). No amino acid side chain of the protein appears to make specific contacts or forms any hydrogen bond with the functional groups of the 3'-adenine (personal communication from Poul Nissen). Therefore, it is probably not the hydrogen bonding pattern on the 3'-terminal base of aminoacyl-tRNA that is recognized by EF-Tu. An analysis of the crystal structure of the ternary complex suggested that the hydrophobic platform in EF-Tu favors a purine base over a pyrimidine one, and the dipole alignment with Glu271 of EF-Tu favors adenine over guanine (Nissen et al., 1996). These results agree well with our observed interactions between 3'-terminal variants of valyl-tRNA^{Val} and EF-Tu:GTP.

Interaction between positions 74 and 75 variants of valyl-tRNA^{Val} and elongation factor Tu

The ability of positions 74 and 75 variants of valyl-tRNA^{Val} to participate in the polypeptide synthesis (Figure 14) indicate that mutants at these two positions are active in all steps of the elongation cycle. As expected, no dramatic effects on ternary complex formation
are observed upon making systematic changes at C74 and C75, as monitored by the hydrolysis protection assay and Kd determinations. EF-Tu:GTP fully protects all tRNA\textsuperscript{Val} mutants at positions 74 and 75 from deacylation (Figures 16 and 17). The Kds of ternary complexes with all tRNA\textsuperscript{Val} mutants at positions 74 and 75 are very similar to the Kd of the complex with wild-type valyl-tRNA\textsuperscript{Val} (Table 8). These results agree well with the crystal structure where only the phosphates of C74 and C75 interact with Lys52 of EF-Tu, while the bases of A73, C74 and C75 are stacked in continuation of the acceptor helix without any specific interaction with the protein (Nissen, 1995). Therefore, all mutations at positions 74 and 75 in this study, as well as many base modifications at these two positions from other studies (Baksht \textit{et al.}, 1977) can be accommodated by EF-Tu in formation of ternary complexes.

This study has shown that the 3'-terminal base of a tRNA is specifically recognized by elongation factor Tu. The amino acid residues in the pocket of EF-Tu that interacts with the 3'-end of aminoacyl-tRNA, discriminate against pyrimidines and only allow purines at the 3'-terminal position. These results agree well with the observation that C76 and U76 valyl-tRNA\textsuperscript{Val} are unable to participate in polypeptide synthesis, while G76 valyl-tRNA\textsuperscript{Val} is an active participant in polypeptide synthesis.
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