Nucleic Acids Research, 1997, Vol. 25, No. 24 **4883–4890**

Role of acceptor stem conformation in tRNA^{Val} recognition by its cognate synthetase

Mingsong Liu[+], Wen-Chy Chu, Jack C.-H. Liu and Jack Horowitz*

Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011, USA

Received September 22, 1997; Revised and Accepted November 3, 1997

ABSTRACT

Although the anticodon is the primary element in *Escherichia coli* tRNA^{Val} for recognition by valyl-tRNA synthetase (ValRS), nucleotides in the acceptor stem and other parts of the tRNA modulate recognition. Study of the steady state aminoacylation kinetics of acceptor stem mutants of *E.coli* tRNA^{Val} demonstrates that replacing any base pair in the acceptor helix with another Watson–Crick base pair has little effect on aminoacylation efficiency. The absence of essential recognition nucleotides in the acceptor helix was confirmed by converting *E.coli* tRNA^{Ala} and yeast tRNA^{Phe}, whose acceptor stem sequences differ significantly from that of tRNA^{Val}, to efficient valine acceptors. This transformation requires, in addition to a valine anticodon, replacement of the G:U base pair in the acceptor stem of these tRNAs. Mutational analysis of tRNA^{Val} verifies that G:U base pairs in the acceptor helix act as negative determinants of synthetase recognition. Insertion of G:U in place of the conserved U4:A69 in tRNA^{Val} reduces the efficiency of amino-acylation, due largely to an increase in K_m. A smaller but significant decline in aminoacylation efficiency occurs when G:U is located at position 3:70; lesser effects are observed for G:U at other positions in the acceptor helix. The negative effects of G:U base pairs are strongly correlated with changes in helix structure in the vicinity of position 4:69 as monitored by ^{19}F NMR spectroscopy of 5-fluorouracil-substituted tRNA^{Val}. This suggests that maintaining regular A-type RNA helix geometry in the acceptor stem is important for proper recognition of tRNA^{Val} by valyl-tRNA synthetase. ^{19}F NMR also shows that formation of the tRNA^{Val}-valyl-tRNA synthetase complex does not disrupt the first base pair in the acceptor stem, a result different from that reported for the tRNA^{Gln}-glutaminyl-tRNA synthetase complex.

INTRODUCTION

Correct aminoacylation of tRNAs by aminoacyl-tRNA synthetases is crucial to maintaining the fidelity and efficiency of protein synthesis. Each synthetase must distinguish its cognate tRNA(s) from structurally similar non-cognate tRNAs. *In vivo* and *in vitro* methods of functional analysis have been developed (reviewed in 1–3) to identify those nucleotides or structural features essential for accurate tRNA recognition and aminoacylation (positive determinants) and those that block recognition of non-cognate tRNAs (negative determinants). Only a limited number of nucleotides contribute to tRNA recognition. Their location in tRNA varies and their distribution and relative contributions to synthetase recognition differ from one tRNA to another. They may be scattered throughout the tRNA molecule as in yeast tRNA^{Phe} (4), yeast tRNA^{Asp} (5), *Escherichia coli* tRNA^{Phe} (6), *E.coli* tRNA^{Arg} (7) and *E.coli* tRNA^{Ser} (8), but are often localized in the first few base pairs of the acceptor stem, e.g., tRNA^{Ala} (9) and tRNA^{His} (10,11), or in the anticodon loop (*E.coli* tRNA^{Met}) (12) or both [*E.coli* tRNA^{Gln} (13) and tRNA^{Gly} (14)].

Major recognition elements essential for correct aminoacylation of *E.coli* tRNA^{Val} by-valyl-tRNA synthetase (ValRS) are located in the anticodon (12,15,16), but nucleotides in the acceptor stem and in other parts of the tRNA (Liu *et al.*, unpublished) may also contribute to synthetase recognition. Computer assisted comparison of *E.coli* and *Salmonella typhimurium* tRNA sequences (17) indicated that one of the distinguishing characteristics of valine-specific tRNAs is a conserved U4:A69 base pair in the acceptor stem. ^{19}F NMR studies of the interaction of ValRS with 5-fluorouracil-substituted tRNA^{Val} (18), and nuclease V1 foot-printing experiments (15, Liu *et al.*, unpublished), have shown that valyl-tRNA synthetase either contacts the U4:A69 base pair directly or induces structural changes in that region of the acceptor stem.

To directly determine the contribution of nucleotides in the acceptor stem of *E.coli* tRNA^{Val}, and in particular of the U4:A69 base pair, to recognition by ValRS, we have analyzed the steady state aminoacylation kinetics of mutant tRNA^{Val} transcripts by purified ValRS. No positive synthetase recognition determinants were found in the acceptor stem. Substituting Watson–Crick base pairs for any base pair in the acceptor stem has little effect on aminoacylation. However, a G:U wobble base pair at any of several positions in the acceptor stem reduces catalytic efficiency. ^{19}F NMR experiments with 5-fluorouracil-substituted tRNA^{Val} ([FUra]tRNA^{Val}) suggest that maintaining regular A-type RNA helix geometry in the acceptor stem is important for proper

*To whom correspondence should be addressed. Tel: +1 515 294 8344; Fax: +1 515 294 0453; Email: jhoro@iastate.edu

[+]Present address: Cardiovascular Research Institute, University of California–San Francisco, San Francisco, CA 94143, USA

recognition of tRNA^{Val} by ValRS. These results are supported by transferring identity elements of tRNA^{Val} into the framework of *E.coli* tRNA^{Ala} and yeast tRNA^{Phe}.

MATERIALS AND METHODS

Transcription of tRNA

Escherichia coli tRNA^{Val} was transcribed *in vitro* (19) from the recombinant phagemid, pVAL119-21, which contains the wild-type *E.coli* tRNA^{Val} gene linked directly to an upstream T7 promoter (19). Methods similar to those described for tRNA^{Val} (19,20) were used to construct a phagemid containing the gene for *E.coli* tRNA^{Ala} (UGC); a plasmid with the yeast tRNA^{Phe} gene (21) was the gift of Dr O.C.Uhlenbeck (University of Colorado). Mutations were introduced into the cloned tRNA genes by site-directed mutagenesis (22) using mutagenic oligonucleotides synthesized by the Nucleic Acids Facility at Iowa State University. Mutants were selected by dideoxy DNA sequence analysis (23). Transcription was catalyzed by T7 RNA polymerase (24) in the presence of 4 mM of each nucleoside-5′-triphosphate [FUTP replaced UTP for synthesis of (FUra)tRNA^{Val}] and 16 mM GMP, to produce tRNA with a 5′-terminal monophosphate (19). AMP replaced GMP in transcription of the tRNA^{Val} variant having a 5′-terminal adenylate; yields of this transcript are low, but sufficient quantities were obtained for aminoacylation assays. The transcripts were purified by HPLC as described earlier (19). Nucleoside triphosphates (ATP, CTP, GTP and UTP) were products of the United States Biochemical Corporation (Cleveland, OH); 5-fluorouridine triphosphate was synthesized by Sierra Biochemicals (Tucson, AZ). Transfer RNA was quantified by spectrophotometric measurements at 260 nm, assuming a value of $E^{0.1\%}_{260} = 24$.

Aminoacylation kinetics

Initial rates of aminoacylation with *E.coli* ValRS, purified to homogeneity (18), were measured at 37°C, in 60 µl reaction mixtures containing 100 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 10 mM KCl, 7 mM ATP, 1 mM DTT, 99 µM [^{3}H]valine (5 Ci/mmol) and 0.5–6.0 µM transfer RNA (determined by measuring valine acceptance at high ValRS concentration). Reactions were initiated by addition of 1 nM ValRS, and 10 µl samples were removed at the indicated times, spotted on Whatman 3MM paper, and processed as described by Bruce and Uhlenbeck (25). Results are averages of at least three experiments. The estimated error of the measurements is ±20%.

^{19}F NMR spectroscopy

For NMR spectroscopy, tRNA samples were dissolved in a minimum volume of standard buffer (50 mM sodium cacodylate, pH 6.0, 15 mM $MgCl_2$, 100 mM NaCl and 1 mM EDTA), and then dialyzed against two changes of the same buffer. The sample volume was then adjusted to 0.405 ml, and 10% (v/v) D_2O was added as an internal lock signal. ^{19}F NMR spectra were collected at 30°C on a Varian Unity 500 FT NMR spectrometer at 470 MHz by using 16K data points, with no relaxation delay and a pulse angle optimizing the Ernst condition (26). ^{19}F chemical shifts are reported downfield from free 5-fluorouracil dissolved in standard buffer.

RESULTS

Absence of synthetase recognition determinants in the acceptor helix of *E.coli* tRNA^{Val}

In many tRNAs, synthetase recognition determinants are located in the acceptor stem (1–3). It has been suggested (16) that the conserved U4:A69 base pair found in bacterial tRNA^{Val} and, to a lesser extent the G3:C70 base pair, serve as minor determinants for ValRS recognition. Our studies of the steady state aminoacylation kinetics of acceptor stem mutants of *in vitro* transcribed *E.coli* tRNA^{Val} show that replacing the conserved U4:A69 with any other Watson–Crick base pair yields tRNA^{Val} variants that are almost as active as wild-type tRNA^{Val} (Table 1). The somewhat low activity of the G4:C69 mutant, relative k_{cat}/K_m = 0.31, may be due to rigidity of the acceptor stem as a result of the five consecutive G:C base pairs in this mutant tRNA^{Val}.

Table 1. Aminoacylation kinetics of acceptor stem variants of *E.coli* tRNA^{Val}

tRNA		K_m (µM)	k_{cat} (sec^{-1})	k_{cat}/K_m	Relative k_{cat}/K_m
Wild-type tRNA^{Val}		1.4	9.0	6.4	(1.0)
G1:C72	→ A1:U72	1.6	9.4	5.9	0.91
	→ A1*C72	1.5	8.3	5.5	0.86
G2:C71	→ C2:G71	1.7	7.7	4.6	0.71
	→ U2:A71	1.6	9.0	5.6	0.87
G3:C70	→ A3:U70	1.5	8.3	5.5	0.86
	→ C3:G70	1.4	7.2	5.1	0.80
U4:A69	→ A4:U69	1.6	8.8	5.5	0.86
	→ C4:G69	1.4	7.7	5.5	0.86
	→ G4:C69	6.2	12.2	2.0	0.31
A6:U67	→ G6:C67	2.3	10.3	4.5	0.69
U7:A66	→ C7:G66	1.6	6.8	4.3	0.67

Table 2. Aminoacylation of *E.coli* $tRNA^{Ala}$ and $tRNA^{Phe}$ variants with valine

tRNA	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m	Relative k_{cat}/K_m
Wild-Type $tRNA^{Val}$(UAC)	1.4	9.0	6.4	(1.0)
***Escherichia coli* $tRNA^{Ala}$**				
$tRNA^{Ala}$(UGC)G3:U70 (wild-type)	No detectable aminoacylation[a]			
$tRNA^{Ala}$(UGC)G3:C70	46.7	0.49	0.01	0.0016
$tRNA^{Ala}$(UGC)A3:U70	–	–	0.0043	0.00067
$tRNA^{Ala}$(UAC)	1.1	0.31	0.51	0.079
$tRNA^{Ala}$(UAC)G3:C70	0.80	4.3	5.4	0.84
$tRNA^{Ala}$(UAC)A3:U70	0.73	5.0	6.9	1.07
Yeast $tRNA^{Phe}$				
$tRNA^{Phe}$(GAA)G4:U69 (wild-type)	–	–	7.0×10^{-5}	1.1×10^{-5}
$tRNA^{Phe}$(GAA)A4:U69	–	–	2.3×10^{-4}	3.6×10^{-5}
$tRNA^{Phe}$(GAC)G4:U69	3.3	3.1	0.93	0.14
$tRNA^{Phe}$(GAC)A4:U69	2.1	7.6	3.6	0.56

[a]Less than 0.02 pmol valine per pmol tRNA.

Substitution for other base pairs in the acceptor helix also has little effect on the kinetics of aminoacylation. Replacing G1:C72 with A1:U72 does not significantly lower the aminoacylation of $tRNA^{Val}$ (Table 1), indicating that the identity of the first base pair plays no appreciable role in $tRNA^{Val}$ recognition by ValRS. Furthermore, it is not essential that the first base be paired; the mutant $tRNA^{Val}$ with an A1*C72 mismatch is an excellent substrate for ValRS (Table 1). Liu *et al.* have reported (27) that G1*A72 and G1*G72 substitutions also have no effect on the rate of $tRNA^{Val}$ aminoacylation. Replacement of G2:C71 by either A2:U71 or C2:G71, and of G3:C70 with either C3:G70 or A3:U70, generates mutant tRNAs with relative k_{cat}/K_m values close to that of wild-type $tRNA^{Val}$ (Table 1). Mutant $tRNA^{Val}$ with Watson–Crick base pair-substitutions for A6:U67 and U7:A66, in the lower part of the acceptor helix, have moderately reduced specificity constants (Table 1), but remain good substrates for ValRS.

Conversion of *E.coli* $tRNA^{Ala}$ and yeast $tRNA^{Phe}$ to valine acceptors

Additional evidence to support the conclusion that the acceptor stem of wild-type $tRNA^{Val}$(UAC) lacks essential synthetase recognition determinants was obtained by converting *E.coli* $tRNA^{Ala}$(UGC) and yeast $tRNA^{Phe}$(GAA) into efficient valine-accepting tRNAs. The acceptor stem sequences of both tRNAs differ considerably from that of $tRNA^{Val}$ (shaded areas in Fig. 1b and c). Because the second and third positions of the anticodon are major synthetase recognition nucleotides of $tRNA^{Val}$ (12,15,16), we first prepared $tRNA^{Ala}$(UAC) and $tRNA^{Phe}$(GAC), in which the alanine and phenylalanine anticodons were changed to valine anticodons.

Wild-type *E.coli* $tRNA^{Ala}$(UGC) is not aminoacylated by ValRS (Table 2). Changing the alanine anticodon to that of valine, creating $tRNA^{Ala}$(UAC), increases valine charging efficiency somewhat (also see ref. 16) but the k_{cat}/K_m of $tRNA^{Ala}$(UAC) is still more than 12 times lower than that of wild-type $tRNA^{Val}$ due to a 16-fold decrease in k_{cat} (Table 2).

Conversion of $tRNA^{Ala}$(UAC) to an even better substrate for ValRS requires replacing the G3:U70 wobble base pair in the acceptor stem with a Watson–Crick base pair (also see ref. 16). The resulting tRNAs, $tRNA^{Ala}$(UAC)G3:C70 and $tRNA^{Ala}$(UAC)A3:U70, are aminoacylated at a rate comparable to that of wild-type $tRNA^{Val}$ (Table 2) despite the absence of U4:A69 and other differences in acceptor stem sequence. Replacing the wobble base pair (G3:U70) in the acceptor stem of $tRNA^{Ala}$ does little to increase valine charging efficiency unless the valine anticodon is also present (Table 2). The results confirm the absence of positive synthetase recognition determinants in the acceptor helix of *E.coli* $tRNA^{Val}$, and also suggest that G:U wobble base pairs in the acceptor stem act as negative determinants of $tRNA^{Val}$ identity.

These conclusions are reinforced by experiments in which yeast $tRNA^{Phe}$(GAA) is transformed into a valine accepting species. This tRNA differs from *E.coli* $tRNA^{Val}$(UAC) at 28 positions; its sequence includes a G:U base pair at 4:69 in the acceptor stem (Fig. 1c). Wild-type yeast $tRNA^{Phe}$(GAA) is a very poor substrate for ValRS (Table 2). Introducing a valine anticodon by substituting C36 for A36, to form $tRNA^{Phe}$(GAC), improves the ability of the tRNA to accept valine (Table 2). The efficiency of $tRNA^{Phe}$(GAC) as a substrate for ValRS is increased further by conversion of the G4:U69 wobble base pair in the acceptor stem to an A4:U69 base pair; $tRNA^{Phe}$(GAC)A4:U69 is quite a good substrate for ValRS (Table 2).

Effect of non-standard base pairs in the acceptor helix on synthetase recognition

Negative effects of G:U base pairs on recognition by ValRS were further characterized by introducing wobble base pairs at several positions in the acceptor stem of *E.coli* $tRNA^{Val}$. The results (Table 3) indicate that the reduction in aminoacylation efficiency depends on the position and orientation of the G:U base pair. It is most pronounced with G:U at position 4:69. Mutants of $tRNA^{Val}$ with G4:U69 and U4:G69 substitutions are, respectively, 40- and 6-fold less efficient as substrates of ValRS than wild-type $tRNA^{Val}$ (Table 3). This is due primarily to significant increases in K_m values. The negative influence of G:U base pairs on aminoacylation activity is also evident when the wobble base pair is positioned at 3:70 (Table 3), and the effect decreases as the wobble base pair is moved further from position 4:69. The G1:U72 variant of $tRNA^{Val}$ is as good a substrate for ValRS as wild-type $tRNA^{Val}$ (Table 3); Liu *et al.* (27) also observed no reduction in the rate of $tRNA^{Val}$ aminoacylation as a result of mutating C72 to U72.

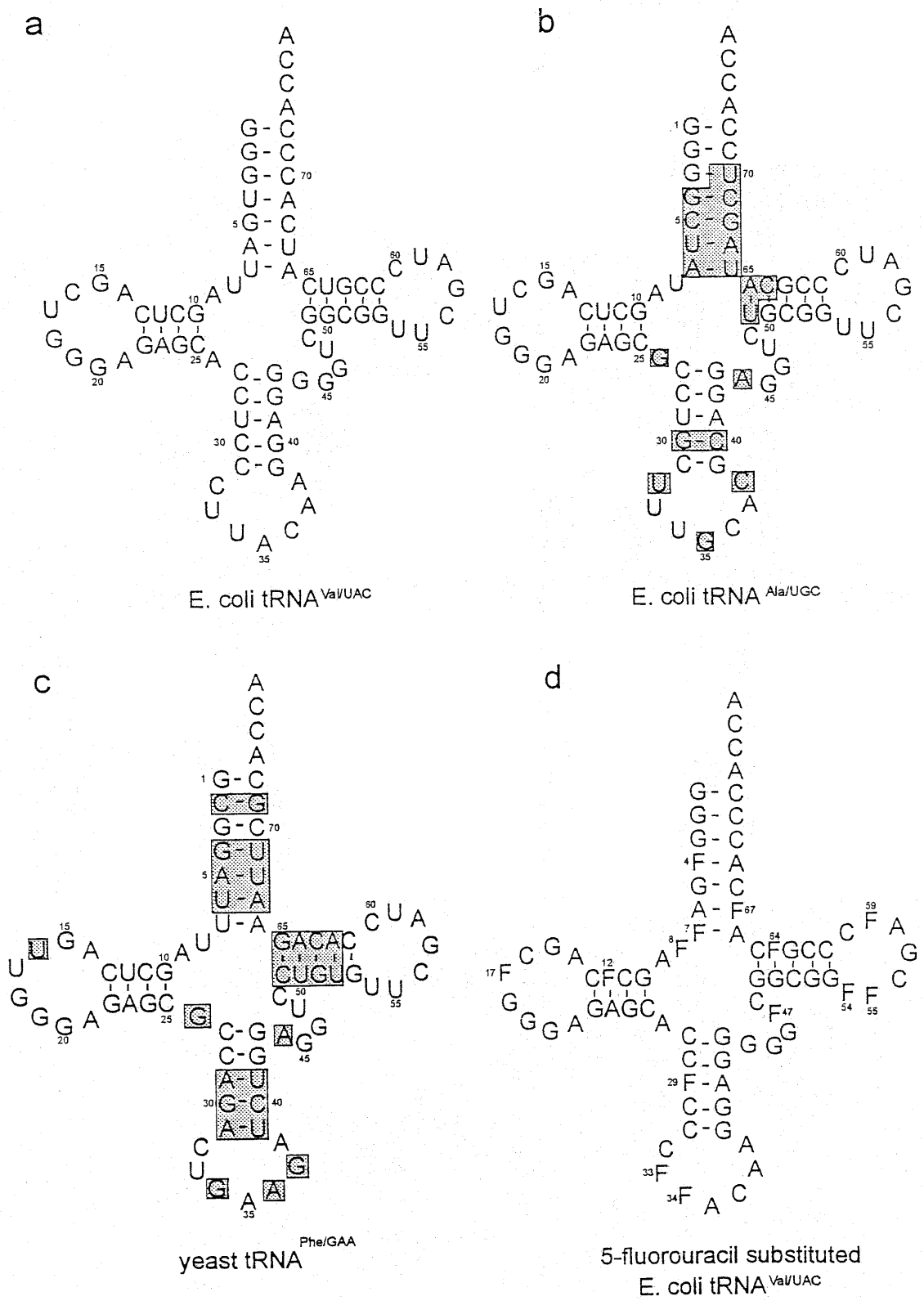

Figure 1. Nucleotide sequence (cloverleaf structure) of the tRNAs used in these studies. (**a**) *Escherichia coli* $tRNA^{Val}$; (**b**) *E.coli* $tRNA^{Ala}$; (**c**) yeast $tRNA^{Phe}$; (**d**) 5-fluorouracil-substituted *E.coli* $tRNA^{Val}$. Shaded areas indicate sequence differences from *E.coli* $tRNA^{Val}$.

Table 3. Aminoacylation kinetics of *E.coli* $tRNA^{Val}$ with noncanonical base pairs in the acceptor stem

tRNA		K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m	Relative k_{cat}/K_m
Wild-Type $tRNA^{Val}$		1.4	9.0	6.4	(1.0)
G1:C72	→ G1:U72	1.5	10.3	6.8	1.06
G2:C71	→ G2:U71	2.7	9.5	3.5	0.55
G3:C70	→ G3:U70	1.8	3.4	1.9	0.30
U4:A69	→ G4:U69	20.0	3.4	0.17	0.027
	→ U4:G69	10.4	11.2	1.1	0.17
	→ U4*C69	2.9	10.4	3.6	0.56
	→ A4*G69	8.9	11.0	1.2	0.19
A6:U67	→ G6:U67	3.2	10.6	3.3	0.52
U7:A66	→ U7:G66	4.1	13.0	3.2	0.50

Base mismatches at the 4:69 position of the acceptor stem also affect aminoacylation. A purine–purine mismatch, A4*G69, decreases aminoacylation activity 5.5-fold, whereas the k_{cat}/K_m for a pyrimidine–pyrimidine mismatch, U4*C69, is closer to that of wild-type $tRNA^{Val}$ (Table 3).

Valyl-tRNA synthetase recognizes acceptor helix structure

To determine whether base mismatches reduce aminoacylation efficiency of $tRNA^{Val}$ by perturbing tRNA structure, the effect of G:U base pairs on acceptor helix geometry was probed by ^{19}F NMR of $tRNA^{Val}$ labeled with fluorine by incorporation of 5-fluorouracil. ^{19}F NMR is ideally suited for this purpose because of the resolution of ^{19}F NMR spectra and the high sensitivity of the fluorine nucleus to changes in its environment. 5-Fluorouracil-substituted $tRNA^{Val}$ retains full aminoacylation activity despite replacement of all uracil residues by the base analog (19,28,29). There are 14 fluorouracil residues distributed throughout every stem and loop of the tRNA molecule (Fig. 1d), and the ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$ shows a resolved peak for every incorporated FUra (19,30,31; Fig. 2a). The spectrum has been completely assigned (32,33) and the assignments are indicated in Figure 2a. ^{19}F signals from fluorouracils paired with guanine resonate 4–5 p.p.m. downfield of those from fluorouracils paired with adenine (19). In the ^{19}F NMR spectra of G:FU-containing (FUra)$tRNA^{Val}$ variants these appear in the region between 6.5 and 7.5 p.p.m. downfield of free FUra (Fig. 2) and are readily assigned by comparison to the spectrum of the wild-type tRNA (Fig. 2a).

The presence of a G:FU base pair in the acceptor helix of (FUra)$tRNA^{Val}$ induces spectral shifts in the ^{19}F NMR spectrum (Fig. 2) in addition to the downfield shift of resonances from fluorouracils paired directly with guanine. These are limited to signals from FUra in the acceptor helix, and presumably reflect structural changes in the acceptor stem that alter the environment of the ^{19}F nucleus in the major groove of the helix. The resonance of FUra in the FU4:A69 base pair is affected most. It shifts downfield when G:U is introduced at positions 2:71 or 3:70 (Fig. 2c and d; Table 4). In the spectrum of $tRNA^{Val}$ mutant G2:FU71 it is shifted 0.39 p.p.m. downfield to partially overlap with FU67; it shifts 1.92 p.p.m. downfield, overlapping with FU7, in the spectrum of mutant G3:FU70. G:U base pairs at positions 1:72, 6:67 and 7:66, have little effect on the chemical shift of resonance FU4. G:FU at 6:67 induces an upfield shift of the resonance corresponding to the adjacent FU7 (Fig. 2e and f; Table 4); FU7 is also shifted upfield by a G:FU base pair at 4:69 (Fig. 2e; Table 4). FU67 is shifted downfield by G:FU at 7:66 (Fig. 2g; Table 4).

There is a strong correlation between the valine accepting activity of $tRNA^{Val}$ variants with G:U base pairs in the acceptor stem and the downfield shift of the FU4 resonance in the ^{19}F NMR spectrum (Table 4). The larger the downfield shift, the lower the aminoacylation efficiency (Table 4). No relationship between aminoacylation efficiency and chemical shift change is observed for other ^{19}F resonances in the acceptor stem.

Substituting A:FU for G:C base pairs in the acceptor helix of (FUra)$tRNA^{Val}$ causes only small changes in the chemical shift position of the FU4 resonance and results in little change in aminoacylation activity of the tRNA (results not shown).

Integrity of the 1:72 base pair on interaction of valyl-tRNA synthetase with $tRNA^{Val}$

The G1:U72 mutant of $tRNA^{Val}$ retains full aminoacylation activity, with a relative specificity constant (k_{cat}/K_m) of 1.06 (Table 3). Incorporation of fluorouracil into this tRNA introduces an additional ^{19}F probe that enables us to examine the effect of ValRS binding on the first (1:72) base pair in the acceptor stem of $tRNA^{Val}$. Crystallographic studies have shown that the terminal U1:A72 base pair of $tRNA^{Gln}$ is disrupted when glutaminyl-tRNA synthetase associates with the tRNA (34,35).

The ^{19}F NMR spectrum of (FUra)$tRNA^{Val}$(G1:U72) shows little difference from the spectrum of wild-type (FUra)$tRNA^{Val}$, except for the additional resonance at 7.31 p.p.m. due to FU72 in the G1:FU72 base pair (compare Fig. 2a and b). ValRS binding to the tRNA causes general line broadening as a result of the longer motional correlation time of the tRNA/ValRS complex (Fig. 3). Spectral changes induced by the enzyme are the same as those observed previously with wild-type $tRNA^{Val}$ (18): loss of intensity of ^{19}F resonances corresponding to FU34, FU7 and FU67 with FU34 being affected most; broadening and shifting of FU12, FU4 and/or FU8 at higher ValRS/tRNA ratios; and a splitting of FU55 and FU64 (Fig. 3). No specific effects on peak FU72 are observed. The corresponding resonance continues to be

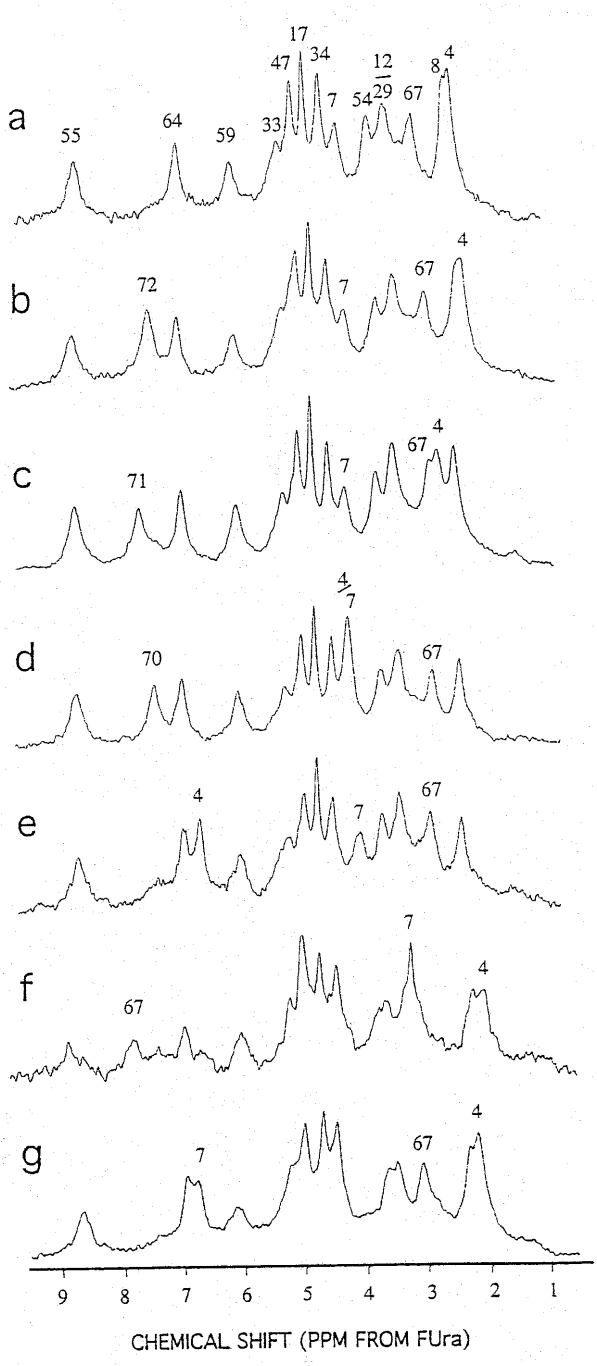

Figure 2. ^{19}F NMR spectra of acceptor stem mutants of 5-fluorouracil-substituted *E.coli* tRNA^{Val}. (**a**) wild-type; (**b**) G1:FU72; (**c**) G2:FU71; (**d**) G3:FU70; (**e**) FU4:G69; (**f**) G6:FU67; (**g**) FU7:G66. Spectra of (FUra)tRNA^{Val} with G:FU base pairs at 4:69, 6:67 and 7:66, obtained under conditions different from those used here, were reported previously (19).

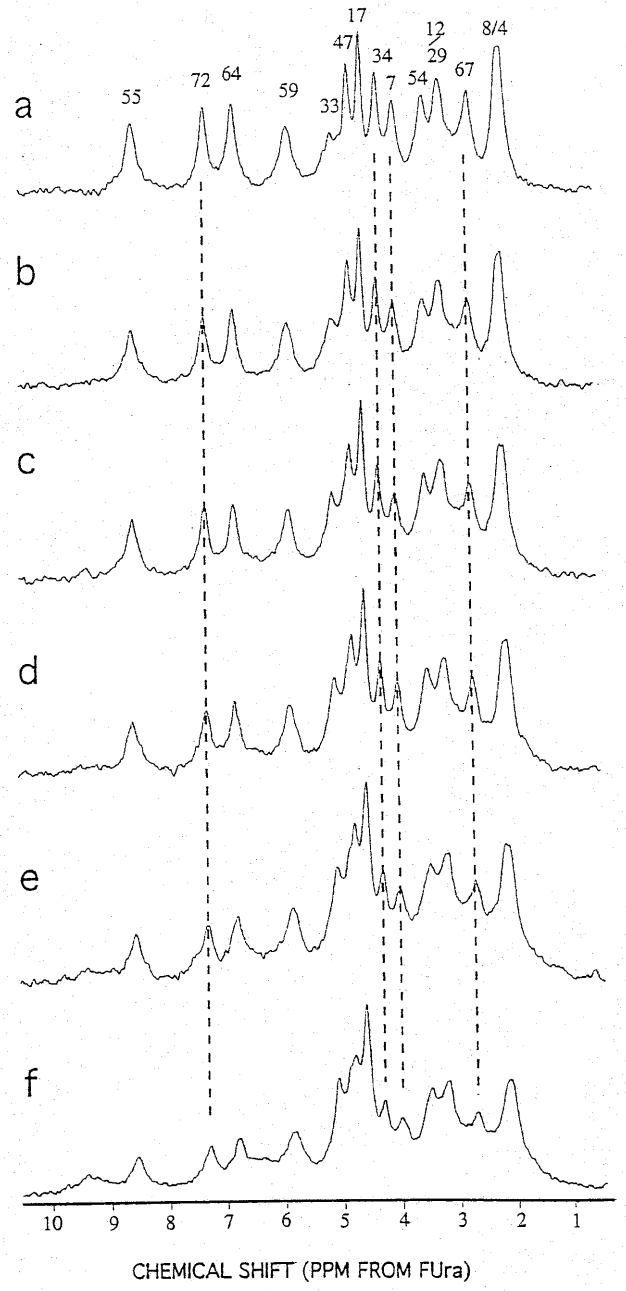

Figure 3. ^{19}F NMR spectra of the 5-fluorouracil-substituted *E.coli* tRNA-^{Val}(G1:U72)-valyl-tRNA synthetase complex. Spectra recorded in the absence of ValRS (**a**) and in the presence of ValRS at a molar ratio to tRNA of: (**b**) 0.25, (**c**) 0.4, (**d**) 0.55, (**e**) 0.75 and (**f**) 0.95.

visible and remains unshifted on addition of increasing amounts of ValRS (Fig. 3), indicating that the first base pair in the acceptor stem remains intact when ValRS interacts with tRNA^{Val}. Opening of the G1:FU72 wobble base pair would shift the FU72 peak upfield to the central region of the ^{19}F NMR spectrum, which has been assigned to resonances from unpaired 5-fluorouracils (32,33).

Table 4. ^{19}F chemical shifts of resonances in the acceptor stem of 5-fluorouracil-substituted *E.coli* valine tRNA variants

tRNA^{Val} variant	Chemical shift (p.p.m.)			δ p.p.m. from wild-type[a]			Relative k_{cat}/K_m[b]
	FU4	FU67	FU7	FU4	FU67	FU7	
Wild-type	2.06	2.70	3.98	0	0	0	(1)
G1:FU72	2.06	2.66	3.97	0	–0.04	–0.01	1.06
G2:FU71	2.45	2.57	3.98	0.39	–0.13	0	0.55
G3:FU70	3.98	2.61	3.98	1.92	–0.09	0	0.30
FU4:G69	6.49	2.68	3.82	4.43	–0.02	–0.16	0.17
G6:FU67	2.01	7.54	3.09	–0.05	4.84	–0.89	0.52
FU7:G66	2.06	2.93	6.64	0	0.23	2.66	0.50

[a]Downfield shift is expressed as a positive number; upfield shift is expressed as a negative number.
[b]For uracil-containing tRNA^{Val}.

DISCUSSION

Although the acceptor helix is the site of identity determinants for many tRNAs, steady state aminoacylation kinetic studies with *E.coli* tRNA^{Val} failed to identify any synthetase recognition nucleotides in this part of the tRNA (Table 1). Aminoacylation efficiency of tRNA^{Val} is not significantly affected by Watson–Crick base pair substitutions at any position in the acceptor helix. The U4:A69 base pair, conserved in bacterial valine-specific tRNAs (17), can be substituted by other Watson–Crick base pairs or even by the pyrimidine–pyrimidine mismatch, U4:C69, with only relatively small decreases in the specificity constant (k_{cat}/K_m) for aminoacylation. The somewhat lower aminoacylation activity of the G4:C69 variant of tRNA^{Val} (Table 1) is probably the result of increased acceptor stem rigidity due to the presence of five consecutive G:C base pairs, rather than to loss of a specific recognition element.

The absence of identity elements in the acceptor helix of tRNA^{Val} was verified by transforming *E.coli* tRNA^{Ala} and yeast tRNA^{Phe} into good substrates for ValRS. Wild-type *E.coli* tRNA^{Ala} and yeast tRNA^{Phe} are very poorly aminoacylated by ValRS (Table 2). Converting the anticodons to a valine anticodon improves valine accepting activity. But only when the G:U base pairs in the acceptor stem are replaced with Watson–Crick base pairs do these tRNAs become good valine acceptors (Table 2), despite the absence of U4:A69 and other differences in acceptor stem sequence. These results support the conclusion that nucleotides in the acceptor helix of tRNA^{Val} are not specifically recognized by ValRS, and suggest that G:U base pairs in the acceptor helix act as negative determinants to prevent proper recognition by ValRS.

Mutational analysis of tRNA^{Val} demonstrates that introduction of G:U wobble base pairs or a purine–purine mismatch into the acceptor stem at or in the vicinity of the 4:69 base pair, at 2:71, 3:70 or 4:69, lowers the aminoacylation efficiency (k_{cat}/K_m) of the tRNA (Table 3). The G4:U69 mutant of tRNA^{Val} is 40 times less efficient as a substrate for ValRs than wild-type tRNA^{Val}, primarily because of an increase in K_m (Table 3); the aminoacylation efficiency of the U4:G69 mutant is also reduced. A A4:G69, purine–purine mismatch, decreases aminoacylation activity significantly (Table 3), whereas a U4:C69, pyrimidine–pyrimidine mismatch, has a smaller effect on activity (Table 3).

It seems likely that G:U wobble and mismatched base pairs inhibit aminoacylation of tRNA^{Val} by distorting the conformation of the acceptor helix. The geometry of a G:U base pair, compared to a standard Watson–Crick base pair, involves displacement of the guanine toward the minor groove. This influences stacking interactions with adjacent base pairs (36,37) in a sequence-dependent manner (38). Crystallographic (36,39,40) and high-resolution NMR (41,42) investigations of tRNAs and short RNA duplexes have shown that G:U base pairs induce local variations in helix geometry at and around the mismatch. Such changes in helical structure are reflected in distinct chemical shift changes in the ^{18}F NMR spectra of G:U-substituted (FUra)tRNA^{Val} (Fig. 2). Introduction of G:U base pairs into the acceptor stem results in shifts of resonances assigned to FUra residues in the acceptor helix. The most prominent spectral change is the downfield shift of the signal from FU4 when G:FU substitutions are made at 2:71, 3:70 and 4:69 (Fig. 2; Table 4). The direct relationship between the magnitude of this shift and the decrease in amino acid accepting activity of the tRNA (Table 4) strongly suggests a correlation between aminoacylation efficiency and acceptor helix conformation in the vicinity of the 4:69 base pair. These results lead us to conclude that ValRS does not specifically recognize the U4:A69 base pair and that an unperturbed A-form helical structure in the middle of the acceptor stem is required for productive interaction of the enzyme with tRNA^{Val}.

Solution of the X-ray structure of the complex of tRNA^{Gln} with glutaminyl-tRNA synthetase, a class I synthetase like ValRS, shows that the 3′ end of the tRNA loops back toward the anticodon, disrupting the 1:72 base pair in the acceptor stem (34,35). Availability of the G1:FU72 variant of (FUra)tRNA^{Val}, which is fully active in aminoacylation (Table 3), permitted us to use ^{19}F NMR to investigate the effect of ValRS binding on the integrity of the acceptor helix of tRNA^{Val}. ^{19}F spectra of this tRNA^{Val} variant show that the 1:72 base pair remains intact as increasing amounts of ValRS bind the tRNA (Fig. 3). Evidently, synthetase-induced disruption of the first base pair in the acceptor stem is not a general characteristic of class I synthetases.

ACKNOWLEDGEMENTS

Support for this investigation was provided by grant MCB 95-13932 from the National Science Foundation. We thank Vahid Feiz for construction of the plasmid containing the gene for *E.coli*

tRNA^{Ala}, Olke Uhlenbeck (University of Colorado, Boulder) for the yeast tRNA^{Phe}-containing plasmid, and Shimin Li and Diane Shogren for technical support. This is Journal Paper No. J-17573 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 3131.

REFERENCES

1 Schulman,L.H. (1991) *Prog. Nucleic Acid Res. Mol. Biol*., **41**, 23–87.
2 Giegé,R., Puglisi,J.D. and Florentz,C. (1993) *Prog. Nucleic Acid Res. Mol. Biol*., **43**, 129–206.
3 McClain,W.H. (1993) *J. Mol. Biol*., **234**, 257–280.
4 Sampson,J.R., DiRenzo,A.B., Behlen,L.B. and Uhlenbeck,O.C. (1989) *Science*, **243**, 1363–1366.
5 Pütz,J., Puglisi,J.D., Florentz,C. and Giegé,R. (1991) *Science*, **252**, 1696–1699.
6 Peterson,E.T. and Uhlenbeck,O.C. (1992) *Biochemistry*, **31**, 10380–10389.
7 McClain,W.H., Foss,K., Jenkins,R.A. and Schneider,J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9260–9264.
8 Normanly,J., Ogden,R.C., Horvath,S.J. and Abelson,J. (1986) *Nature*, **321**, 213–219.
9 Hou,Y.-M. and Schimmel,P. (1988) *Nature*, **333**, 140–145.
10 Himeno,H., Hasegawa,T.,Ueda,T., Watanabe,K., Miura,K.I. and Shimizu,M. (1989) *Nucleic Acids Res.*, **17**, 7855–7863.
11 Franklyn,C. and Schimmel,P. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8655–8659.
12 Schulman,L.H. and Pelka,H. (1988) *Science*, **242**, 765–768.
13 Jahn,M., Rogers,M.J. and Söll,D. (1991) *Nature*, **352**, 258–260.
14 McClain,W.H., Foss,K., Jenkins,R.A. and Schneider,J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 6147–6151.
15 Derrick,W.B., Feiz,V., Chu,W.-C. and Horowitz,J. (1991) *FASEB J*., **5**, A808.
16 Tamura,K., Himeno,H., Asahara,H., Hasegawa,T. and Shimizu,M. (1991) *Biochem. Biophys. Res. Commun*., **177**, 619–623.
17 McClain,W.H. and Nicholas,H.B.,Jr (1987) *J. Mol. Biol*., **194**, 635–642.
18 Chu,W.-C. and Horowitz,J. (1991) *Biochemistry*, **30**, 1655–1663.
19 Chu,W.-C. and Horowitz,J. (1989) *Nucleic Acids Res*., **17**, 7241–7252.
20 Sampson,J. and Uhlenbeck,O.C. (1988) *Proc. Natl. Acad. Sci. USA*, **83**, 1033–1037.
21 Sampson,J.R., Behlen,L.B., DiRenzo,A.B. and Uhlenbeck,O.C. (1992) *Biochemistry*, **31**, 4161–4167.
22 Taylor,J.W., Ott,J. and Eckstein,F. (1985) *Nucleic Acids Res*., **13**, 8765–8785.
23 Sanger,F., Coulson,A.R., Barrell,B.G., Smith,A.J.H. and Roe,B.A. (1980) *J. Mol. Biol*., **143**, 161–178.
24 Grodberg,J. and Dunn,J.J. (1988) *J. Bacteriol*., **170**, 1245–1253.
25 Bruce,G.A. and Uhlenbeck,O.C. (1982) *Biochemistry*, **21**, 855–861.
26 Shaw,D. (1976) *Fourier Transform NMR Spectroscopy*. Elsevier, New York, NY, p. 179.
27 Liu,H., Kessler,J., Peterson,R. and Musier-Forsyth,K. (1995) *Biochemistry*, **34**, 9795–9800.
28 Horowitz,J., Ou,C.-N., Ishaq,M., Ofengand,J. and Bierbaum,J. (1974) *J. Mol. Biol*., **88**, 301–312.
29 Ofengand,J., Bierbaum,J., Horowitz,J., Ou,C.-N. and Ishaq,M. (1974) *J. Mol. Biol*., **88**, 313–325.
30 Hardin,C.C., Gollnick,P., Kallenbach,N.R., Cohn,M. and Horowitz,J. (1986) *Biochemistry*, **25**, 5699–5709.
31 Hardin,C.C., Gollnick,P. and Horowitz,J. (1988) *Biochemistry*, **27**, 487–495.
32 Chu,W.-C., Feiz,V., Derrick,W.B. and Horowitz,J. (1992) *J. Mol. Biol*., **227**, 1164–1172.
33 Chu,W.-C., Kintanar,A. and Horowitz,J. (1992) *J. Mol. Biol*., **227**, 1173–1181.
34 Rould,M.A., Perona,J.J., Söll,D. and Steitz,T.A. (1989) *Science*, **246**, 1135–1142.
35 Rould,M.A., Perona,J.J. and Steitz,T.A. (1991) *Nature*, **352**, 213–218.
36 Mizuno,H. and Sundaralingam,M. (1978) *Nucleic Acids Res*., **5**, 4451–4461.
37 Limmer,S. (1997) *Prog. Nucleic Acid Res. Mol. Biol*., **57**, 1–39.
38 Moras,D., Dumas,P. and Westhof,E. (1986) In van Knippenberg,P.H. and Hilbers,C.W. (eds), *Structure and Dynamics of RNA*. Plenum Press, New York, NY, pp. 113–124.
39 Kim,S.H., Suddath,F.L., Quigley,G.J., McPherson,A., Sussman,J.L., Wang,A.H.J., Seeman,N.C. and Rich,A. (1974) *Science*, **185**, 435–440.
40 Robertus,J.D., Ladner,J.E., Finch, J.T., Rhodes, D., Brown, R.S., Clark,B.F.C. and Klug,A. (1974) *Nature*, **250**, 546–551.
41 Limmer,S., Reif,B., Ott,G., Arnold,L. and Sprinzl,M. (1996) *FEBS Lett.*, **385**, 15–20.
42 Ramos,A. and Varani,G. (1997) *Nucleic Acids Res.*, **25**, 2083–2090.