19F NMR of 5-fluorouracil-substituted transfer RNA transcribed in vitro: resonance assignment of fluorouracil-guanine base pairs

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Received June 26, 1989; Revised and Accepted August 10, 1989

ABSTRACT
5-Fluorouracil is readily incorporated into active tRNAVal transcribed in vitro from a recombinant phagemid containing a synthetic E. coli tRNAVal gene. This tRNA has the expected sequence and a secondary and tertiary structure resembling that of native 5-fluorouracil-substituted tRNAVal, as judged by 19F NMR spectroscopy. To assign resonances in the 19F spectrum, mutant phagemids were constructed having base changes in the tRNA gene. Replacement of fluorouracil in the T-stem with cytosine, converting a FU-G to a C-G base pair, results in the loss of one downfield peak in the 19F NMR spectrum of the mutant tRNAVal. The spectra of other mutant tRNAs having guanine for adenine substitutions that convert FU-A to FU-G base pairs all have one resonance shifted 4.5 to 5 ppm downfield. These results allow assignment of several 19F resonances and demonstrate that the chemical shift of the 19F signal from base-paired 5-fluorouracil differs considerably between Watson-Crick and wobble geometry.

INTRODUCTION
Nuclear magnetic resonance (NMR), because of its high resolution, has been used for studies of the structure and dynamics of transfer RNA in solution. These investigations have employed a number of NMR techniques involving protons and other magnetic nuclei, including 13C, 31P, and 15N (reviewed in 1). Another useful NMR label for the study of biological systems is fluorine-19, and we have employed 19F NMR as a structural probe of tRNAs labeled by incorporation of 5-fluorouracil (FUrA) (2—8). The 19F nucleus has the advantages of 100% abundance, a large range of chemical shifts, and high sensitivity (83% that of 1H). It should be particularly useful as a probe of protein-RNA complexes, such as between tRNA and its cognate synthetase, because the protein makes no contribution to the 19F spectrum. 5-Fluorouracil-substituted tRNAs retain their physical properties despite a high FUrA content (9) and, with few exceptions (10), remain fully functional in protein synthesis in vitro (11), making them ideal for 19F NMR studies. The incorporated fluorine nuclei serve as site-specific reporter groups distributed throughout the stems and loops of the tRNA molecule for monitoring biochemically important conformational changes.

Spectra of three purified 5-fluorouracil-substituted E. coli tRNAs, tRNAVal, tRNArMet and tRNAmMet, have been reported (2, 3, 8), and these show resolved resonances for each of the incorporated FUrA residues. Oligonucleotide binding, chemical modification, and nuclear Overhauser effect studies were used to assign several of the 14 peaks in the 19F NMR spectrum of tRNAVal (4, 5, 7, 8). To verify and complete the assignments, we have prepared FUrA-substituted tRNAVal by in vitro transcription of a synthetic E. coli tRNAVal gene, using a method similar to that recently reported by Sampson and Uhlenbeck (12).
In this paper we describe some of the properties of the fluorouracil-containing in vitro transcript and of several mutant tRNAs with base substitutions for individual FUra residues. Comparison of the $^{19}$F NMR spectra of wild type and mutant (FUra)tRNA$^{Val}$ shows a large downfield $^{19}$F chemical shift change when a fluorouracil-adenine (FU-A) base pair is converted to a fluorouracil-guanine (FU-G) base pair. These shifts permit assignment of several resonances in the $^{19}$F NMR spectrum of (FUra)tRNA$^{Val}$.

MATERIALS AND METHODS

Bacteriophage M13KO7 and phagemid pUC119 were gifts of Dr. Alan Myers, Iowa State University. Oligonucleotides were synthesized on a Biosearch 8750 DNA synthesizer by the Nucleic Acid Facility at Iowa State University. New England Biolabs, Bethesda Research Laboratories, or American Allied Biochemical supplied the restriction endonucleases. T4 DNA ligase and T4 RNA ligase were from Bethesda Research Laboratories and Pharmacia Biochemicals, respectively. T7 RNA polymerase was prepared from E. coli BL21/pAR1219 (13). E. coli tRNA$^{Val}$ was purchased from Subriden RNA, and unfractionated E. coli tRNA was from Plenum Scientific. Nucleoside-5'-triphosphates were supplied by United State Biochemicals, except for 5-fluorouridine-5'-triphosphate (FUTP), which was prepared by Sierra Bioresearch. $[^{14}]$CValine (90 Ci/mole) was purchased from ICN, and $[^{3}]$Hvaline from Amersham. 5-Fluorouracil was a gift from Hoffmann-La Roche Inc.

Construction and cloning of the tRNA$^{Val}$ gene

The E. coli tRNA$^{Val}$ gene, joined directly to an upstream T7 RNA polymerase promoter and a downstream BstNl restriction site and flanked by an EcoRI site at one end and a BamHI site at the other, was prepared by ligation of six synthetic oligodeoxynucleotides (Figure 1) as described by Sampson and Uhlenbeck (12). The resulting 105-bp DNA was purified by electrophoresis on a 6% polyacrylamide gel and ligated into the EcoRI/BamHI site of the phagemid vector pUC119 to produce the recombinant phagemid pVAL119-21. The ligation mixture was used directly to transform E. coli TG1. Clones containing the recombinant plasmid were selected by screening for the 105-bp EcoRI/BamHI insert; identity of the insert was confirmed by dideoxy DNA sequencing (14).

Oligonucleotide-directed mutagenesis

Site-directed mutagenesis of the cloned tRNA$^{Val}$ gene was performed by the method of Eckstein (15, 16). Mutagenic oligonucleotides were synthesized by the Nucleic Acid Facility and single-stranded DNA templates were prepared by infecting E. coli harboring recombinant pUC119 with helper phage M13K07 (17). Mutants were selected by DNA sequence analysis. Mutant tRNAs are designated by the letter and position number of the base in the wild-type sequence that has been altered followed by the base that has been introduced, e.g., tRNA$^{Val}$-A6G has the adenine at position 6 replaced by guanine.

In vitro transcription

DNA templates used to transcribe wild-type and mutant tRNA$^{Val}$ genes were purified by cesium chloride density gradient centrifugation (18) and digested with BstN1 to provide a linear template. The transcription reaction contained 80 μg/ml BstN1 digested DNA, 4 mM of each nucleoside-5'-triphosphate (FUTP replaced UTP for the synthesis of 5-fluorouracil-substituted (tRNA$^{Val}$), 16 mM GMP, 40 mM Tris-HCl, pH 8.1, 22 mM MgCl$_2$, 2 mM spermidine, 5 mM DTT, 50 μg/ml BSA, 80 units/μl of T7 RNA polymerase and 4 units/ml of inorganic pyrophosphatase. After incubation for 2 hr at 42°C, the reaction was stopped by addition of EDTA to a final concentration of 50 mM, and the mixture was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol.
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(25:24:1) and then with chloroform/isoamyl alcohol (24:1). Finally, nucleic acids were ethanol precipitated and dissolved in water. The tRNA transcript was purified from contaminating nucleoside triphosphates, products of incomplete transcription, and DNA template by chromatography on a Toyopearl DEAE-650S column (12).

The rate of transcription is approximately the same with UTP and FUTP. A typical transcription reaction yields 55—60 nmol of tRNA per ml, as determined by valine acceptance, corresponding to 1.4—1.6 mg active tRNA\textsuperscript{Val} per ml. Yields of the fluorinated transcript are slightly lower, about 1.2 mg per ml.

**Aminoacylation of tRNA**

Kinetic measurements were carried out at 37°C in 65 μl reactions containing 100 mM HEPES, pH 7.5, 15 mM MgCl\textsubscript{2}, 10 mM KCl, 7 mM ATP, 1 mM DTT, and 99 μM \[^3\text{H}\]valine (5.0 Ci/mmol). Transfer RNA concentrations ranged from 0.3 to 2.7 μM, determined by calculating the valine acceptance, and reactions were initiated by addition of 1.2 nM purified valyl-tRNA synthetase. Ten μl were removed at 30-s intervals, spotted on Whatman 3MM paper, and processed as described by Bruce and Uhlenbeck (19). \(K_M\) and \(V_M\) were determined by a linear least-square fit of the double-reciprocal plot of the data (20).

Valyl-tRNA synthetase was purified to homogeneity (unpublished procedure) from an overproducing strain of \textit{E. coli}, GRB 238, carrying the plasmid pHOV1 (21) kindly supplied by Dr. George Marchin, Kansas State University.

**Fluorine-19 NMR Spectroscopy**

For \(^{19}\text{F}\) NMR spectroscopy, transfer RNA was dissolved in a minimum volume of buffer containing 55.55 mM sodium cacodylate, pH 6.0, 16.66 mM MgCl\textsubscript{2}, 111 mM NaCl, and 1.11 mM EDTA. After dialysis against two changes of 250 ml of the same buffer in a flow-dialysis microcell (BRL model 1200 MA), the sample volume was adjusted to 0.3 ml, and 10% (v/v) D\textsubscript{2}O was added to serve as an internal deuterium lock. The tRNA was renatured by heating at 55°C for 20 min and slowly cooled to room temperature before transfer to a Wilmad 529A-10 spherical NMR microcell. This was suspended in a 10-mm NMR tube and surrounded with sample buffer containing 1 mM 5-fluorouracil as an external standard. To remove Mg\textsuperscript{2+} for studies at low divalent cation concentrations, tRNA samples were treated with EDTA as described previously (4).

\(^{19}\text{F}\) NMR spectra were obtained at 282 MHz on a Bruker WM-300 pulsed FT NMR spectrometer at room temperature. Spectra were accumulated using 8K data points, no relaxation delay, and a pulse width sufficient to optimize the Ernst condition (22), except when determining integrals, in which case a 5-s relaxation delay and a 90° pulse width were employed to ensure complete relaxation. Chemical shifts are reported as ppm from free 5-fluorouracil; downfield shifts are indicated as positive.

**RESULTS**

In vitro synthesis of tRNA\textsuperscript{Val}

**Construction of plasmid and in vitro transcription.** The recombinant phagemid pVAL119-21, containing an \textit{E. coli} tRNA\textsuperscript{Val} gene linked directly to a T7 RNA polymerase promoter, was constructed by ligating six synthetic oligonucleotides (Figure 1) and cloning the resulting 105-bp DNA fragment into the EcoRI/BamHI site of pUC119 (see Methods). 5-Fluorouracil is readily incorporated into tRNA\textsuperscript{Val} when BstNI-digested pVAL119-21 is transcribed by T7 RNA polymerase in the presence of 5-fluorouridine-5'-triphosphate. Electrophoresis of the transcription product on a 20% polyacrylamide/8M urea gel shows a single major
RNA component that has the same mobility as native normal and native FUra-substituted tRNAVal isolated from E. coli cells; the uracil-containing in vitro transcript migrates somewhat more slowly (results not shown).

Characteristics of the in vitro transcript. Synthetic tRNAVal is a faithful copy of the DNA sequence, judged by the position of the guanosine residues in a partial RNase T1 digest ladder derived from 5' [32P]-labeled transcript (not shown). Furthermore, the transcript has the expected 5'-terminal pppGp, as demonstrated by the detection of [32P]-labeled pppGp when the RNase T2 digestion products of tRNAVal labeled by synthesis in the presence of [α-32P]GTP are analyzed by two-dimensional thin layer chromatography (TLC). Transcripts prepared in a reaction primed with excess GMP (GMP/GTP=4) have 5'-terminal guanosine monophosphate.

The 3'-end of synthetic tRNAVal is heterogeneous, presumably due to non-template-directed incorporation of one or more nucleotides (12, 23, 24). Seventy five percent of the transcripts possess the expected 3'-terminal adenosine, as determined by two-dimensional TLC of a complete RNase T2 hydrolyzate of RNA 3'-end labeled with 5'-[32P]pCp by T4 RNA ligase; 20% terminate in C, 4% in G, and 1% in U. The transcription products are separated into two bands by electrophoresis on 12% thin polyacrylamide gels under denaturing conditions. Ninety percent of the faster migrating band has a 3'-terminal A residue, whereas the slower migrating RNA band terminates in A (46%), C (44%), G (8%), and U (2%).

Table 1. Aminoacylation Kinetics

<table>
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<th>tRNA</th>
<th>Apparent $K_M$ (μM)</th>
<th>$V_{Max}$ (μmol·min$^{-1}$·mg$^{-1}$)</th>
<th>$V_{Max}/K_M$</th>
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<tr>
<td>Native tRNAVal</td>
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<tr>
<td>Native (FUra)tRNAVal</td>
<td>1.4</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Transcribed (FUra)tRNAVal</td>
<td>1.2</td>
<td>3.8</td>
<td>3.2</td>
</tr>
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</table>
Figure 2. Cloverleaf structure of *E. coli* tRNA<sub>Val</sub> with uracil and uracil-derived bases replaced by 5-fluorouracil (F). The structure includes the two base modifications, m<sup>7</sup>G and m<sup>6</sup>A, found in native (FUra)tRNA<sub>Val</sub> but absent in the in vitro transcript.

In vitro transcribed normal and 5-fluorouracil-substituted tRNA<sub>Val</sub> are both readily aminoacylated by purified valyl-tRNA synthetase (VRS). Table 1 shows that the kinetic parameters of VRS for the normal and fluorinated transcripts differ little from each other and from those for the corresponding native tRNAs isolated from *E. coli* cells. Fluorine-19 NMR spectra of in vitro transcribed (FUra)tRNA<sub>Val</sub> 5-Fluorouracil-substituted tRNA<sub>Val</sub> contains 14 FUra residues (Figure 2). A signal from each of the incorporated fluorines can be resolved in the <sup>19</sup>F NMR spectrum of the fluorinated synthetic tRNA<sub>Val</sub> (Figure 3b). Comparison of the spectrum of the fluorinated in vitro transcript with that of native (FUra)tRNA<sub>Val</sub>, isolated from FUra-treated *E. coli* and having ca. 95% of its uracil and uracil-derived minor bases replaced by 5-fluorouracil (Figure 3a and b), shows that the two spectra are almost identical when recorded at high Mg<sup>2+</sup> (15 mM), suggesting that there are few if any significant structural differences between synthetic and native (FUra)tRNA<sub>Val</sub> under these conditions. The few spectral differences include a 0.36 ppm shift of peak E, so that it now appears downfield of D, and a 0.36 ppm upfield shift of peak K in the spectrum of the in vitro transcript. Small downfield shifts of peaks F and H are also observed (Figure 3). Peaks have been designated as described for the <sup>19</sup>F spectrum of native (FUra)tRNA<sub>Val</sub> (4). Corresponding resonances are identified on the basis of peak assignments using several different strategies including base replacement (8 and unpublished observations). The downfield shift of resonance A in the spectrum of tRNA transcribed in vitro probably is due to small differences in ionic strength or magnesium ion concentration because the chemical shift position of this <sup>19</sup>F signal is known to vary greatly with solution conditions (4, 8).

In the absence of magnesium, the <sup>19</sup>F NMR spectrum of the in vitro transcript differs
Figure 3. $^{19}$F NMR spectra of 5-fluorouracil-substituted tRNA$^{\text{Val}}$. (a) Native tRNA$^{\text{Val}}$ isolated from 5-fluorouracil-treated E. coli; (b) in vitro tRNA$^{\text{Val}}$ transcript; (c) native tRNA$^{\text{Val}}$ isolated from 5-fluorouracil-treated E. coli; (d) in vitro tRNA$^{\text{Val}}$ transcript. Spectra (a) and (b) were obtained in 15 mM Mg$^{2+}$, at 47°C, while (c) and (d) were recorded in the absence of added Mg$^{2+}$ at 22°C.

markedly from that of native tRNA$^{\text{Val}}$ (compare Figure 3c and d). This is especially true in the high field region where resonances in the spectrum of tRNA transcribed in vitro have lost intensity and are less well defined. There is an increase in the relative intensity of peaks in the central region of the spectrum, where signals from fluorouracils in relatively unstructured, loop regions of the tRNA are known to resonate (4, 8).

Table 2. Mutagenic Primers and Mutants Produced

<table>
<thead>
<tr>
<th>Mutagenic Primer</th>
<th>Mutant</th>
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<tr>
<td>$5'\text{-TATAGGGTGGTTAGC-3'}$</td>
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</tr>
<tr>
<td>$5'\text{-CAAGGGGGGGGTCGGCGG-3'}$</td>
<td>A41G</td>
</tr>
<tr>
<td>$5'\text{-GGTTCGATCCCGCCATC-3'}$</td>
<td>F64C</td>
</tr>
<tr>
<td>$5'\text{-CCCGTCGTCACCCAC-3'}$</td>
<td>A66G</td>
</tr>
<tr>
<td>$5'\text{-CGTCATCGCCCAACCA-3'}$</td>
<td>A69G</td>
</tr>
</tbody>
</table>

*Indicates position of the mutation
Figure 4. $^{19}$F NMR spectrum of a T-stem mutant of fluorinated tRNA$^{Val}$. (a) Wild-type 5-fluorouracil-substituted tRNA$^{Val}$; (b) mutant tRNA$^{Val}$ with F64 replaced by C. Spectra were recorded at 22°C.

Peak assignments in the $^{19}$F NMR spectrum of 5-fluorouracil-substituted tRNA$^{Val}$

Site-directed mutagenesis of valine tRNA. To assign resonances in the $^{19}$F NMR spectrum of (FUra)tRNA$^{Val}$ we have compared the spectrum of the wild-type tRNA with those of transcripts carrying substitutions for individual FUra residues. Mutations were introduced into the tRNA$^{Val}$ gene by the oligonucleotide-directed mutagenesis method described by Eckstein and coworkers (15, 16). The mutagenic primers used are shown in Table 2; mismatched bases are indicated by an asterisk. The sequence of each mutant was confirmed by dideoxy DNA sequencing.

Assignment of 5-fluorouracil 64, in the T-stem. Replacement of FU64 with cytosine converts the G50-FU64 base pair in the T-stem of (FUra)tRNA$^{Val}$ (Figure 2) to a G-C base pair. Comparison of the $^{19}$F NMR spectrum of (FUra)tRNA$^{Val}$-F64C with the spectrum of wild-type (FUra)tRNA$^{Val}$ (Figure 4) shows that they are identical except for the absence of peak B at 6.67 ppm in the spectrum of the mutant, and a small downfield shift of the resonance at 3.27 ppm (peak I). This permits direct assignment of peak B to FU64 and corrects the previous tentative assignment, based on indirect evidence, of peak B to FU54 (8). The global structure of (FUra) tRNA$^{Val}$ is not perturbed by the change of FU to C at position 64, as judged by the $^{19}$F NMR spectrum.

Assignment of 5-fluorouracil 29, in the anticodon stem. Assignment of FU64, which is base paired with G50, to peak B in the downfield region of the $^{19}$F NMR spectrum of (FUra)tRNA$^{Val}$ is surprising in view of our earlier results suggesting that the $^{19}$F signal from 5-fluorouracils hydrogen-bonded in helical stems of tRNA resonate in the upfield region of the $^{19}$F NMR spectrum (4). To determine whether the downfield chemical shift
position of the resonance from FU64 is characteristic of 5-fluorouracils involved in base pairing with guanine, variant tRNAs containing other G-FU base pairs were prepared.

Introduction of a G-FU base pair into the anticodon stem of (FUra)tRNA\textsuperscript{Val}, by replacing A41 with G, yields a tRNA whose \textsuperscript{19}F NMR spectrum, recorded at 47°C, differs from that of wild-type (FUra)tRNA\textsuperscript{Val} in the shift of one resonance, identified as K, from 2.7 ppm, where it appears as a low-field shoulder on peak L (Figure 5a), downfield to 7.7 ppm (Figure 5b); an additional minor change is the upfield shift of peak M to overlap peak N.

\textsuperscript{19}F NMR spectra of mutant A41G obtained at room temperature confirm the identity of the peak shifting downfield as K. In spectra of wild-type (FUra)tRNA\textsuperscript{Val} taken at the lower temperature (22°C), peak K overlaps peak J at 2.9 ppm (Figure 5c). Spectra of mutant A41G recorded at 22°C show that one of the two resonances in peak J/K shifts to 7.7 ppm (Figure 5d). Clearly, the peak at 7.7 ppm in the spectrum of (FUra)tRNA\textsuperscript{Val}-A41G is due to a 4.6–4.8 ppm downfield shift of resonance K as a consequence of FU29 pairing with G (at position 41) rather than A. These results allow assignment of peak K to FU29.

5-Fluorouracils in the acceptor stem. The acceptor stem of (FUra)tRNA\textsuperscript{Val} contains three fluorouracil residues base paired with adenine (Figure 2). Replacement of A with G results, in each case, in the downfield shift of one peak in the \textsuperscript{19}F NMR spectrum (Figure 6). Mutant A6G, in which the A6-FU67 base pair is replaced by G6-FU67, yields a spectrum
with peak L shifted from 2.5 ppm to 7.4 ppm (Figure 6b); a smaller upfield shift of peak G to partly overlap peak I is also observed.

In the $^{19}$F NMR spectra of mutants A69G, which has G69 base paired with FU4, and A66G, in which G66 is base paired with FU7, a new downfield peak is observed at 6.2—6.4 ppm (Figure 6c and d). This is the result of a downfield shift of peak N in the spectrum of mutant A69G; peak G again shows a small upfield shift. The resonance shifted in the spectrum of mutant A66G is difficult to identify (see Discussion).

On the basis of these observations, we can assign peak L to FU67, and peak N to FU4.

**DISCUSSION**

The use of $^{19}$F NMR spectroscopy to probe tRNA structure and the recognition of tRNA by its cognate aminoacyl-tRNA synthetase requires, among other things, the ready availability of large amounts of purified FUra-substituted tRNAs and the assignment of fluorine resonances in the $^{19}$F spectra of these tRNAs. Both these needs can be met by using the approaches described here. Our results demonstrate that 5-fluorouridine is readily incorporated into tRNA synthesized in vitro by T7 RNA polymerase-catalyzed transcription of a synthetic *E. coli* tRNA\textsubscript{Val} gene. High yields and ease of purification make this the method of choice for the preparation of fluorinated tRNAs for $^{19}$F NMR studies.

Characterization of the tRNA\textsubscript{Val} transcribed in vitro indicates that it closely resembles...
native tRNA\textsuperscript{Val} except for some 3’-end heterogeneity; 25% of the transcript contains one or two extra 3’-terminal nucleotides. This nontemplate directed incorporation of nucleotides is also observed by others (12, 23, 24) and seems to be characteristic of bacteriophage RNA polymerase-catalyzed transcription in vitro (25). The kinetics of valine acceptance by transcribed normal and FUra-substituted tRNA\textsuperscript{Val} are quite similar to each other and to those of the native tRNA species (Table 1).

At high magnesium ion concentrations, in vitro transcribed (FUra)tRNA\textsuperscript{Val} and that synthesized in vivo have very similar secondary and tertiary structures, as shown by the similarity of their \textsuperscript{19}F NMR spectra (Figure 3a and b). Because the chemical shift of the fluorine nucleus is very sensitive to changes in tRNA conformation (4–8), any significant structural variations would be reflected in spectral changes. The small chemical shift changes of peaks E and K in the spectrum of the fluorinated in vitro transcript may be due to the absence of m\textsuperscript{7}G and m\textsuperscript{6}A in this tRNA; these are the only two modified bases present in native (FUra)tRNA\textsuperscript{Val} prepared from \textit{E. coli} cells (9).

In the absence of Mg\textsuperscript{2+}, the \textsuperscript{19}F NMR spectrum of (FUra)tRNA\textsuperscript{Val} transcribed in vitro differs from that of native (FUra)tRNA\textsuperscript{Val} (Figure 3), suggesting that the secondary or tertiary structure of the transcript is less stable at low divalent cation concentrations than that of the native tRNA. Sampson and Uhlenbeck (12) reported that yeast tRNA\textsuperscript{Phe} transcribed in vitro has a lower T\textsubscript{m} than native tRNA\textsuperscript{Phe}. These authors attribute the reduced thermal stability to an altered binding of Mg\textsuperscript{2+} ions due to the absence of modified bases in the in vitro transcript. However, native (FUra)tRNA\textsuperscript{Val}, which lacks all but two (m\textsuperscript{6}A\textsubscript{37} and m\textsuperscript{7}G\textsubscript{46}) of the modified bases found in native normal tRNA\textsuperscript{Val} (9), has a T\textsubscript{m} very similar to that of the latter (9). It seems that the absence of modified bases cannot entirely explain the instability of the in vitro transcript and further investigation is required to clarify the factors involved.

The relative ease of preparing mutant tRNAs from the cloned tRNA\textsuperscript{Val} gene by oligonucleotide-directed mutagenesis permits us to systematically assign resonances in the \textsuperscript{19}F NMR spectrum of (FUra)tRNA\textsuperscript{Val} by selectively replacing individual FUra residues. The first such replacement examined involved FU\textsubscript{64}, which is hydrogen-bonded to G\textsubscript{50} in the T-stem of the (FUra)tRNA\textsuperscript{Val} molecule (Figure 2). Absence of the resonance at 6.67 ppm, peak B, from the spectrum of mutant F\textsubscript{64}C (Figure 4) allows ready assignment of peak B to FU\textsubscript{64}. The assignment of a resonance in the downfield region of the spectrum to a fluorouracil located in a hydrogen-bonded, helical region of the tRNA, differs from expectations based on our previous results. Consideration of ring-current effects and the preferential perturbation of upfield \textsuperscript{19}F resonances by the cyclophotoaddition of 4’-(hydroxymethyl)-4,5’,8-trimethyl-psoralen, which is known to react most readily with pyrimidines in double-stranded regions, led us to initially assign 5-fluorouracils in helical stems to the upfield resonances in the \textsuperscript{19}F NMR spectrum of (FUra)tRNA\textsuperscript{Val} (2, 4).

Assignment of FU\textsubscript{64} to peak B suggests that signals from FUra residues involved in base pairing with G resonate in the downfield region of the \textsuperscript{19}F spectrum. This conclusion is supported by experiments in which four other A-FU base pairs were individually converted to G-FU base pairs: FU\textsubscript{29}-A\textsubscript{41} in the anticodon stem; A\textsubscript{6}-FU\textsubscript{67}, FU\textsubscript{4}-A\textsubscript{69}, and FU\textsubscript{7}-A\textsubscript{66} in the acceptor stem. Each of these substitutions results in a 4- to 5-ppm downfield shift of a single peak from the 1.5- to 3.2-ppm region of the \textsuperscript{19}F spectrum of (FUra)tRNA\textsuperscript{Val} to the 6- to 8-ppm range (Figures 5 and 6). The downfield shift of peak K in the \textsuperscript{19}F NMR spectrum of mutant A41G due to formation of the F29-G41 base pair (Figure 5) allows assignment of K to FU\textsubscript{29}. Peak L in the spectrum
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of mutant A6G, shifts downfield when A6-FU67 is replaced by G6-FU67 (Figure 6b), permitting assignment of L to FU67. Similarly, peak N can be assigned to FU4 on the basis of the downfield shift of this resonance in the spectrum of mutant A69G (Figure 6c).

Assignment of FU7, which is base-paired to residue 66, is not straightforward. The spectrum of mutant A66G shows one resonance from the upfield region shifted downfield (Figure 6d). At first glance it appears that peak L has shifted, however, this resonance has already been assigned to FU67. Closer inspection of the mutant A66G spectrum indicates that resonance M may have shifted downfield and peak L shifted 0.5 ppm upfield to the position usually occupied by M; peak G also appears to shift upfield, as it does in the spectra of the other acceptor-stem mutants of (FUra)tRNAVal (Figure 6). Further study is required to confirm this conclusion, but a shift of peak L (FU67) might be expected if M corresponds to FU7, because the two 5-fluorouracil residues are located at adjacent base pairs in the acceptor stem of (FUra)tRNAVal (Figure 2). A base change at one position, especially the introduction of a G-U base pair at the end of the acceptor helix, may affect the environment of the fluorine at the adjacent base pair.

The reasons for the large difference in the 19F chemical shift between FU-A and FU-G base pairs are not clear. Several groups have examined the nature of the bonding in FU-G base pairs in DNA oligomers. Kremer et al. (26) have suggested that because of the low pKa of 5-fluorouracil, ca. 7.8, a large fraction of FUra in a DNA duplex heptamer exists in the ionized form at physiological pH. Involvement of the ionized form of the fluoropyrimidine in the FU-G base pair could account for at least part of the observed downfield chemical shift, because dissociation of the N3 imino proton of FUra results in a downfield shift of the 19F resonance (4). Other investigators, however, have reported (27, 28, 29) that the apparent pKa of the FUra imino proton in base-paired structures is greater than 9.5, considerably higher than the value reported by Kremer et al. (26). Furthermore, we have shown (4) that the chemical shift of peak B (FU64-G50) in the 19F NMR spectrum of (FUra)tRNAVal does not change in the pH range 4.5–8.5. These results effectively rule out an ionized structure for FUra in FU-G base pairs in tRNA.

X-ray crystallographic (30) and NMR (28) studies of several DNA oligomer duplexes in which thymine is replaced by 5-fluorouracil show that the FU-G base pair has wobble geometry. No evidence for either the ionized or enol tautomeric form of fluorouracil is found in these structures. The difference in stacking geometry of Watson-Crick (FU-A) and wobble (FU-G) base pairs, due to the different functional groups of FUra involved in these structures, significantly changes the environment of the fluorine nucleus in the major groove of the helix (28), and presumably accounts for the large downfield chemical shift of resonances from FU-G base pairs in the 19F NMR spectra of (FUra)tRNAVal.

ACKNOWLEDGEMENTS

We thank Lian Li for assistance in the preparation of the synthetic tRNAVal gene, Vahid Feiz and Wes Derrick for the preparation of several mutant tRNAs, Jeff Sampson and Olke Uhlenbeck for communicating details of their transcription system before publication, Deb Stowers and Jenny Keim for synthesis of oligonucleotides in the Nucleic Acid Facility of Iowa State University, Drs. F.W. Studier (State University of New York at Stony Brook) and G. Marchin (Kansas State University) for the E. coli strains overproducing T7 RNA polymerase and valyl-tRNA synthetase, respectively, and Dr. P.E. Sorter of Hoffmann La Roche, Nutley, N.J., for a generous supply of 5-fluorouracil.

Journal Paper No. J-13572 of the Iowa Agriculture and Home Economics Experiment
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