19F NMR studies of 5-fluorouracil-substituted
Escherichia coli transfer RNAs: solution structure
and codon-anticodon interaction

Paul Dennis Gollnick
Iowa State University

Follow this and additional works at: http://lib.dr.iastate.edu/rtd
Part of the Biochemistry, Biophysics, and Structural Biology Commons

Recommended Citation
Gollnick, Paul Dennis, "19F NMR studies of 5-fluorouracil-substituted Escherichia coli transfer RNAs: solution structure and codon-
anticodon interaction " (1986). Retrospective Theses and Dissertations. 8002.
http://lib.dr.iastate.edu/rtd/8002

This Dissertation is brought to you for free and open access by Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This reproduction was made from a copy of a manuscript sent to us for publication and microfilming. While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. Pages in any manuscript may have indistinct print. In all cases the best available copy has been filmed.

The following explanation of techniques is provided to help clarify notations which may appear on this reproduction.

1. Manuscripts may not always be complete. When it is not possible to obtain missing pages, a note appears to indicate this.

2. When copyrighted materials are removed from the manuscript, a note appears to indicate this.

3. Oversize materials (maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or in black and white paper format.*

4. Most photographs reproduce acceptably on positive microfilm or microfiche but lack clarity on xerographic copies made from the microfilm. For an additional charge, all photographs are available in black and white standard 35mm slide format.*

*For more information about black and white slides or enlarged paper reproductions, please contact the Dissertations Customer Services Department.
Gollnick, Paul Dennis

FLUORINE-19 NMR STUDIES OF 5-FLUOROURACIL-SUBSTITUTED ESCHERICHIA COLI TRANSFER RNAs: SOLUTION STRUCTURE AND CODON-ANTICODON INTERACTION

Iowa State University

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages
2. Colored illustrations, paper or print
3. Photographs with dark background
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages √
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received
16. Other

University Microfilms International
$^{19}$F NMR studies of 5-fluorouracil-substituted *Escherichia coli* transfer RNAs: Solution structure and codon-anticodon interaction

by

Paul Dennis Gollnick

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved:
Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy

For the Major Department
Signature was redacted for privacy.
For the Graduate College

Iowa State University
Ames, Iowa
1986
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABBREVIATIONS</strong></td>
<td>v</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>Function of tRNA</td>
<td>1</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>1</td>
</tr>
<tr>
<td>Other roles of tRNA</td>
<td>4</td>
</tr>
<tr>
<td><strong>General Structural Features of tRNA</strong></td>
<td>5</td>
</tr>
<tr>
<td>Primary structure</td>
<td>5</td>
</tr>
<tr>
<td>Modified nucleosides</td>
<td>8</td>
</tr>
<tr>
<td>Three dimensional structure of yeast tRNA&lt;sub&gt;Phe&lt;/sub&gt;</td>
<td>10</td>
</tr>
<tr>
<td>Solution structure of tRNA</td>
<td>12</td>
</tr>
<tr>
<td><strong>Dynamics of tRNA</strong></td>
<td>13</td>
</tr>
<tr>
<td>Conformational states in solution</td>
<td>13</td>
</tr>
<tr>
<td><strong>Codon-Anticodon Interaction</strong></td>
<td>15</td>
</tr>
<tr>
<td>Conformational changes</td>
<td>16</td>
</tr>
<tr>
<td><strong>NMR Studies of tRNA</strong></td>
<td>19</td>
</tr>
<tr>
<td>Assignment of proton resonances</td>
<td>20</td>
</tr>
<tr>
<td>Melting</td>
<td>20</td>
</tr>
<tr>
<td>Ion binding to tRNA</td>
<td>21</td>
</tr>
<tr>
<td>Interaction with proteins</td>
<td>22</td>
</tr>
<tr>
<td><strong>Incorporation of 5-Fluorouracil into Transfer RNA</strong></td>
<td>23</td>
</tr>
<tr>
<td><strong>&lt;sup&gt;19&lt;/sup&gt;F NMR Studies of FUra-Substituted tRNA</strong></td>
<td>25</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL PROCEDURES</strong></td>
<td>31</td>
</tr>
<tr>
<td>Materials</td>
<td>31</td>
</tr>
</tbody>
</table>
### Methods

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth of <em>E. coli</em> in the presence of 5-fluorouracil</td>
<td>33</td>
</tr>
<tr>
<td>tRNA preparation</td>
<td>33</td>
</tr>
<tr>
<td>Purification of FUra-substituted tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>34</td>
</tr>
<tr>
<td>Purification of FUra-substituted tRNA&lt;sub&gt;Val&lt;/sub&gt;</td>
<td>35</td>
</tr>
<tr>
<td>Purification of FUra-substituted tRNA&lt;sub&gt;Val&lt;/sub&gt;</td>
<td>35</td>
</tr>
<tr>
<td>Aminoacylation assay</td>
<td>35</td>
</tr>
<tr>
<td>Formylation assay</td>
<td>36</td>
</tr>
<tr>
<td>Analysis of nucleoside composition</td>
<td>36</td>
</tr>
<tr>
<td>Polyacrylamide gel electrophoresis</td>
<td>38</td>
</tr>
<tr>
<td>5' End labeling tRNA with &lt;sup&gt;32&lt;/sup&gt;P</td>
<td>38</td>
</tr>
<tr>
<td>Aniline cleavage of tRNA</td>
<td>39</td>
</tr>
<tr>
<td>Ribonuclease H reactions</td>
<td>40</td>
</tr>
<tr>
<td>Limited ribonuclease T1 digestion of tRNA</td>
<td>40</td>
</tr>
<tr>
<td>Bisulfite modification of FUra-substituted tRNA&lt;sub&gt;Val&lt;/sub&gt;</td>
<td>41</td>
</tr>
<tr>
<td>Photoreaction of psoralen with FUra-containing tRNA</td>
<td>41</td>
</tr>
<tr>
<td>Oligonucleotide synthesis</td>
<td>42</td>
</tr>
<tr>
<td>NMR spectroscopy</td>
<td>45</td>
</tr>
</tbody>
</table>

### RESULTS

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification and Partial Characterization of FUra-substituted tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>48</td>
</tr>
<tr>
<td>Purification of (FUra)tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>48</td>
</tr>
<tr>
<td>Nucleoside analysis of (FUra)tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>49</td>
</tr>
<tr>
<td>&lt;sup&gt;19&lt;/sup&gt;F NMR spectra of FUra-substituted tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>53</td>
</tr>
<tr>
<td>Determination of the position of fluorodihydrouridine in (FUra)tRNA</td>
<td>57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;sup&gt;19&lt;/sup&gt;F NMR Studies of the Solution Structure of FUra-substituted tRNA</td>
<td>60</td>
</tr>
<tr>
<td>Effect of pH on the &lt;sup&gt;19&lt;/sup&gt;F NMR of FUra-substituted tRNA</td>
<td>61</td>
</tr>
<tr>
<td>&lt;sup&gt;19&lt;/sup&gt;F NMR studies of ion binding to FUra-substituted tRNA</td>
<td>65</td>
</tr>
<tr>
<td>Temperature dependence of the &lt;sup&gt;19&lt;/sup&gt;F NMR spectrum of (FUra)tRNA&lt;sub&gt;Val&lt;/sub&gt;</td>
<td>82</td>
</tr>
<tr>
<td>Reaction of FUra-substituted tRNA&lt;sub&gt;Val&lt;/sub&gt; with bisulfite</td>
<td>85</td>
</tr>
</tbody>
</table>
Photoreaction of 4'-(hydroxymethyl)-4,5',8-trimethyl psoralen with FUra-substituted tRNA\textsubscript{Val} 87

19F NMR Studies of Codon-Anticodon Interaction in FUra-substituted tRNA 88
- Effect of codon-anticodon interaction on the 19F NMR spectrum of (FUra)tRNA\textsubscript{Val} 90
- Characterization of the effect of the 3'-terminal A in GpUpApA 98
- Formation of an anticodon-anticodon complex 104
- Evidence that the codon-containing oligonucleotide bind to the anticodon loop 104
- Influence of magnesium and spermine on the codon-anticodon interaction 110
- Codon-anticodon interaction in FUra-substituted methionine tRNAs 118
- 19F NMR study of possible conformational change resulting from codon-anticodon interaction 123

DISCUSSION 126
- Examination of the Effects of Solution Conditions and Chemical Reactivity of the 19F Resonances in the Spectra of FUra-Substituted tRNA 129
- Ion binding to FUra-substituted tRNA 134

Codon Anticodon Interaction in FUra-Substituted tRNAs 138

REFERENCES 150

ACKNOWLEDGMENTS 166
ABBREVIATIONS

FUra (F), 5-fluorouracil
NMR, nuclear magnetic resonance
ppm, parts per million
RNA, ribonucleic acid
DEAE, diethylyaminoethyl
BD-cellulose, benzoylated DEAE-cellulose
EDTA, ethylenediaminetetraacetic acid
Tris, 2-amino-2-hydroxymethyl-1,3-propanediol
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
bicine, N,N-bis (2-hydroxyethyl) glycine
TEAB, triethylammonium bicarbonante
s^U, 4-thiouridine
ψ, pseudouridine
rT, ribothymidine
m^A, 6-methyladenosine
m^G, 7-methylguanosine
NOE, nuclear Overhauser effect
Hz, hertz
HMT, 4'-hydroxymethyl-4,5',8-trimethyl psoralen
INTRODUCTION

A commonly held belief in biochemistry is that a knowledge of structure will lead to a better understanding of function. This has held true for many systems. When Perutz (1952) solved the three-dimensional structure of hemoglobin it paved the way for numerous advances in understanding the functions of this molecule. The mechanisms of many enzymes have been understood, at least in part, through determining the arrangement of amino acids in their active sites. The crystal structure of yeast tRNA°° has been known for more than ten years (Kim et al., 1974), and the primary sequences of over 200 tRNAs are currently known (Gauss and Sprinzl, 1985). This structural knowledge has provided a firm foundation for further study; however, we are still far from understanding the relationship of structure to the functions and specificity of transfer RNAs.

In this study, 19F nuclear magnetic resonance was used to investigate the solution structure and dynamics of transfer RNA substituted with the uracil analog 5-fluorouracil. Effects of solutions conditions on the 19F NMR spectra were studied, and a detailed investigation of codon-anticodon interaction was made. The eventual goal of these studies is to relate the structural conformation of tRNA to the important biological functions of this molecule in the transmission of genetic information during protein synthesis.

Function of tRNA

Protein synthesis

Transfer RNA is central to protein synthesis in all living systems. It recognizes a codon triplet of the messenger RNA and provides the amino acid specified by the codon to the growing polypeptide chain. In this
way, transfer RNA acts as the adaptor molecule in the flow of genetic information first hypothesized by Francis Crick (quoted in Hoagland, 1960) several years before the discovery of tRNA. A brief description of the role of tRNA in bacterial protein synthesis follows, for a more detailed review of this subject see Pongs (1978).

Aminoacylation To function in protein synthesis, tRNA must first be aminoacylated. Twenty different aminoacyl-tRNA-synthetases, corresponding to the 20 different amino acids in biological systems, esterify the carboxyl group of an amino acid to the 2' or 3' hydroxyl group of the terminal adenosine of its cognate tRNA(s) in an ATP requiring reaction. Frequently several tRNAs, termed isoacceptors, exist for a given amino acid.

The essential feature of this system is a high degree of specificity. Because these enzymes must differentiate one species of tRNA from another with high fidelity, all tRNAs must have elements of uniqueness. Despite many years of investigation, the structural features of tRNA involved in determining the specificity of aminoacylation are not known precisely. Rich and Schimmel (1977) have proposed a model in which synthetases interact with tRNAs along the inside of the two branches of the L-shaped tRNA molecule, and contacts with the anticodon arm are important. However, recent studies have indicated that this generalization may not hold true for all tRNAs (Romby et al., 1985).

Initiation Protein synthesis begins with N-formylmethionyl tRNA^Met binding to the initiation codon of the mRNA (AUG or GUG) on the small (30S) ribosomal subunit. This process requires GTP and three initiation factors, IF-1, IF-2, and IF-3. The resulting 30S initiation complex then reacts with the 50S ribosomal subunit, GTP is hydrolyzed, and the initiation factors are released to yield a 70S initiation complex with fMet-tRNA^Met bound at the P-site on the ribosome.
**Elongation**  
Elongation of the polypeptide chain occurs by codon-dependent binding of aminoacyl tRNAs to the A-site of the ribosome as a ternary complex with the elongation factor, EF-Tu, and GTP. A peptide bond is formed between the amino group of the aminoacyl-tRNA in the A-site and the carboxyl group of the fMet or nascent peptide in the P-site, through the catalytic activity of the peptidyl transferase center in the 50S ribosomal subunit. The energy for peptide-bond formation comes from hydrolysis of the "energy rich" acyl-ester bond of the aminoacyl-tRNA at the P-site, which in turn is derived from ATP hydrolyzed during aminoacylation. The free tRNA at the P-site is released and the peptidyl-tRNA is then translocated from the A-site to the P-site. After translocation, one cycle of elongation has been completed. The vacant A-site now contains a new mRNA codon, to which a corresponding aminoacyl-tRNA can bind and start another round of elongation.

**Termination**  
The elongation process continues until one of the three termination codons, UAA, UGA, or UAG, appears in the A-site. At this point, under the influence of three specific proteins called releasing factors (RF-1, RF-2, RF-3), the ester linkage of the peptidyl-tRNA is hydrolyzed, releasing the completed polypeptide. The deacylated tRNA and mRNA are then expelled from the ribosome.

During protein synthesis, tRNA is involved in several functions and interacts with numerous proteins and nucleic acids. Some of these interactions require exquisite specificity, such as with aminoacyl-tRNA-synthetases. Others, such as the formation of a ternary complex with EF-Tu and GTP, involve a group of tRNAs, and all tRNAs must be sufficiently alike to function in the same ribosomal apparatus during protein synthesis. Therefore, tRNAs must have elements of uniqueness and yet maintain a certain degree of similarity. The mechanism by which an enzyme or protein recognizes a specific tRNA or a group of tRNAs is not yet clear.
Other roles of tRNA

Transfer RNA is involved in many biological functions other than protein synthesis. Aminoacyl-tRNAs are involved in regulating several bacterial amino acid biosynthetic operons through attenuation (Yanofsky, 1981). The tryptophan (trp) operon of E. coli is the best characterized of these systems. Expression of this operon is sensitive to the relative levels of charged and uncharged tRNATrp (Bertrand et al., 1975). The mechanism of regulation involves coupling transcription with translation of a short peptide coding region in the leader transcript while RNA polymerase pauses transcription at a specific site (Yanofsky et al., 1983). The leader peptide coding region contains two tandem trp codons. Ribosome stalling at either of these codons, caused by a deficiency of trp-tRNATrp, promotes formation of a transcript secondary structure which prevents transcription termination and the operon is expressed. However, if sufficient trp-tRNATrp is present to allow translation of the entire leader region, an alternative secondary structure is formed which causes termination of transcription (attenuation).

Transfer RNAs also function as primers for DNA synthesis by reverse transcriptase of RNA tumor viruses (Dahlberg, 1980). Tryptophan tRNA is the primer for avian virus reverse transcriptase (Waters et al., 1975) whereas tRNA^Phe functions with the murine enzyme (Dahlberg, 1980). Other tRNAs may also be used by other viruses (Peters and Dahlberg, 1979).

In enteric bacteria, tRNA, in conjunction with other components of the protein synthetic apparatus plays, a major role in metabolic regulation through the stringent response to shut off RNA synthesis when protein synthesis is stopped (Cashel and Gallert, 1974). This regulation occurs, for example, when cells are subjected to amino acid starvation. When mRNA and deacylated tRNA are bound to the ribosome, the magic spot compounds pppGpp and ppGpp are synthesized and the cells adjust their metabolism by
ceasing ribosomal RNA synthesis (Cashel, 1969).

Several special tRNAs also transfer amino acids in non-ribosomal dependent reactions, such as the tRNA\textsubscript{Gly} required for \textit{Staph. epidermidus} cell wall biosynthesis (Roberts, 1972). More detailed information on the diverse roles of transfer RNA in cellular functions has been presented in a review by Larossa and Soll (1978).

General Structural Features of tRNA

**Primary structure**

Transfer RNAs are polynucleotide chains, typically 75-90 nucleotides in length with an average molecular weight of 26,000. The first sequence of a tRNA molecule, yeast alanine tRNA, was announced in 1965 by R. Holley and seven co-workers (Holley et al., 1965). They noted that the polynucleotide chain could be arranged into a cloverleaf secondary structure to optimize the intrastrand Watson-Crick base-pairing. Presently, over 200 tRNAs have been sequenced (Gauss and Sprinzl, 1985), and all cytoplasmic tRNAs can be arranged into this general cloverleaf pattern depicted in Figure 1A.

A generalized structure of tRNA (Fig. 1A) consists of the amino acid acceptor stem, and three arms: the D arm, T arm, and anticodon arm, each composed of a stem and loop structure. The acceptor stem contains the 5' and 3' ends of the tRNA, and terminates at the 3' end in the conserved sequence CpCpA. During aminoacylation, the cognate amino acid is esterified to the ribose of the 3'-terminal adenosine. The D and T arm are so named because their loops invariably contain the modified nucleosides dihydrouridine (D) and ribothymidine (T). The anticodon arm contains the three anticodon nucleotides that play a direct role in the transfer of genetic information through interaction with codon-triplets in the messenger RNA.
Figure 1. Structural features of transfer RNA.
(A) Generalized cloverleaf structure of tRNA. Invariant and semi-invariant residues are designated by capital letters. R is purine, Y is pyrimidine, and H is hypermodified purine. Major landmarks on the structure are indicated.
(B) Two views of a schematic diagram of the tertiary structure of yeast tRNA^Phe. The sugar-phosphate backbone of the molecule is represented as a tube with cross-rungs showing the nucleotide base-pairs. Dark circles represent four magnesium hydrate ions and "spaghetti" represents spermine molecules.
As a rule, the number of nucleotides in stems and loops of tRNA is constant. The acceptor stem contains seven nucleotide-pairs, the T and anticodon stems each contain five base-pairs each, and three or four base-pairs are found in the D stem. Seven nucleotides are usually found in the T and anticodon loops. Two variable regions, alpha and beta, occur in the D loop (Fig. 1A), each containing one to three nucleosides in addition to the five constant purine residues found in this loop. The largest site of size variability among tRNAs is the variable (V) loop, which consists of four or five nucleosides in most tRNAs (class I) but can contain up to 21 nucleosides (class II).

A number of positions in the cloverleaf sequence are occupied by constant (invariant) nucleotides, as indicated in Figure 1A. Other positions are semi-invariant, occupied exclusively by purines (R), pyrimidines (Y), or hypermodified nucleosides (H). Most of the invariant and semi-invariant nucleotides are found in the loop regions of the molecule (Fig. 1A). The general features and implications of the primary and secondary structure of tRNA are discussed in a review by Clark (1978).

Several tRNAs, usually those having special functions, deviate slightly from the cloverleaf shown in Figure 1A (Clark, 1978). Only mitochondrial tRNAs are known to show large deviations from this general structure (Roe et al., 1981). Sequence data from bovine mitochondrial tRNAs demonstrate that, with the exception of tRNA\textsuperscript{Leu}, all of the 22 species lack features commonly found in other tRNAs. The extreme case is tRNA\textsuperscript{Ser}, which lacks the entire D arm (DeBruijn et al., 1980).

Modified nucleosides

An interesting aspect of the primary structure of tRNA is the presence of nucleosides other than the four normally found in RNA. More than 50 different modified nucleosides have been isolated and characterized
These modifications range from simple methylation of the base, or 2'-O of ribose, to complex hypermodified nucleosides. The extensive degree of nucleotide modification in tRNA is one of the unique features of these molecules.

Most of these nucleosides result from post transcriptional enzymatic modification of a major nucleotide in the tRNA molecule. However, at least one modification, guanine to queosine (Q), takes place at the nucleoside level (Agris and Soll, 1977). One enzyme generally modifies a given residue at a specific site in all tRNAs; $m^5$U-methylase, for example, catalyzes the synthesis of ribothymidine in the T loop of all E. coli tRNAs (Greenberg and Dudock, 1980). However, the same modification at different sites in a tRNA may involve several enzymes, as is the case in pseudouridine synthesis (Green et al., 1982).

Modified nucleosides are usually located at specific positions in the cloverleaf structure of tRNA; starred nucleotides in Figure 1A are ones that are usually modified. For example, ribothymidine (T) and pseudouridine ($\psi$) are almost always found at positions 54 and 55. Hypermodified nucleosides are often found in the first position of the anticodon, or 3'-adjacent to it.

Their complex structures, specificity of location in the molecule, and presence in tRNA from all organisms suggests that modified nucleosides play an important role in the function of tRNA. Several functions proposed for modified bases include affects on the stability (Nishimura, 1977) and specificity (Yokoyama et al., 1985) of codon-anticodon interaction, and protecting tRNA from attack by ribonuclease (Roe et al., 1973). Modified bases may also be involved in protein recognition of tRNA (Kim, 1978). However, despite intensive research in this area, there is presently no real proof of an important universal role of modified nucleosides in tRNA function. Indeed, several studies have shown that some tRNAs
remain fully functional without certain modifications (Ramabhadran et al., 1976; Horowitz et al., 1974).

**Three dimensional structure of yeast tRNA^{Phe}**

**Overall structure**  A schematic illustration of the three-dimensional structure of yeast tRNA^{Phe}, as determined by X-ray crystallography, is shown in Figure 1B (Kim et al., 1974; Sussman et al., 1978). The molecule is L-shaped, with the anticodon loop at one end of the L, and the amino acid acceptor stem at the other end. Each extension of the L is about 60 Å long and about 20 Å in diameter. The hydrogen bonding pattern in the cloverleaf (Fig. 1A) is maintained in the three-dimensional structure. When the polynucleotide backbone folds the amino acid stem and the T stem form one continuous double helical arm, and the D stem and the anticodon stem form the other long double helical arm of the L.

The T and D loops meet at the corner of the molecule, and tertiary interactions between these loops stabilize the molecular conformation. Several of these tertiary interactions involve invariant or semi-invariant residues (Figure 1B and 1A), and most involve non-Watson-Crick type interactions such as the reversed Hoogsteen base-pair m^1A58-T54, and the base-triplet G22-C13-m^7G46. Base stacking is the major stabilizing feature of the molecule (Quigley and Rich, 1976) and involves all but 5 of the 76 nucleotides in tRNA^{Phe}; the unstacked bases are located in the variable regions of the D loop, in the variable loop, and in the 3' terminus.

**The anticodon loop**  The conformation of the anticodon loop is similar to that suggested by Fuller and Hodgson (1967). Five bases, including the anticodon triplet, are stacked on the 3' side of the loop (Fig. 1B). A sharp U-turn separates these bases from the invariant uridine-33 located 5' to the anticodon. This conformation is stabilized by several factors including a hydrogen bond from N3 of U-33 to the
oxygen of phosphate-36 and a magnesium ion coordinated to a phosphate oxygen of residue 37. The significant feature of the anticodon is that the bases are stacked in a right-handed helical array which points outward from the loop (Fig. 1B), and presumably is ready to interact with the codon.

**Magnesium and spermine**  The importance of metal ions and cationic polyamines in stabilizing the three-dimensional structure of tRNA has long been recognized (reviewed by Teeter et al., 1980). Four distinct magnesium ions have been identified in the crystal structure of yeast tRNA\(^{\text{Phe}}\) (Fig. 1B) (Quigley et al., 1978). All are found in nonhelical regions and serve to stabilize the loops and turns of the tRNA structure. Two of these ions are located in the D loop, one in the anticodon loop, and one in the sharp turn formed by residues 8 through 12.

Two spermine molecules are found bound to tRNA\(^{\text{Phe}}\) (Fig. 1B). One, located in the major groove at the end of the anticodon stem draws the two opposite ribose phosphate backbones closer together and stabilizes a 25° kink between the anticodon stem and the D stem (Quigley et al., 1978). The second spermine is found near the variable loop and stabilizes a sharp turn in the polynucleotide chain.

**Structure of other tRNAs**  The finding that most of the tertiary interactions in tRNA\(^{\text{Phe}}\) involve conserved or semi-conserved bases suggests that all tRNAs have the same general structural frame (Klug et al., 1974). The crystal structures of four other transfer RNA molecules have been investigated: E. coli tRNA\(^{\text{Met}}\) (Woo et al., 1980), yeast tRNA\(^{\text{Gly}}\) (Wright et al., 1979), yeast initiator methionine tRNA (Sussman and Podjarny, 1983), and aspartic acid tRNA from yeast (Westhof et al., 1985). In all cases, the general L-shaped conformation seen with tRNA\(^{\text{Phe}}\) is observed. However, in several cases interesting subtle conformational differences exist.

In tRNA\(^{\text{Met}}\) from E. coli, a different conformation of the anticodon
loop is observed (Woo et al., 1980). The constant uridine-33 base projects away from the loop, whereas in yeast tRNA^{Phe} it points into the loop (Sussman et al., 1978, Fig 1B). The different loop conformations lead to slightly different stacking arrangements of the anticodon bases in the two tRNAs. These observations have led to speculation that the conformation of the anticodon loop may be a factor in distinguishing the initiator tRNA from elongating tRNAs, and it may have implications concerning conformational changes in the anticodon loop during protein synthesis (Woo et al., 1980).

The three-dimensional structure of yeast tRNA^{Asp} revealed that in the crystal, the tRNA molecules are associated through their partially self-complementary anticodons (GUC). Analysis of the thermal vibrational motions of the tRNA^{Asp} crystal structure revealed a stabilization of the anticodon arm and a destabilization of the T and D loops compared to tRNA^{Phe}. The labilization of the interactions between the T and D loops is suggested to be a consequence of the interaction of the anticodon triplets in tRNA^{Asp}, and may reflect the conformational state of tRNA interacting with messenger RNA (Westhof et al., 1985).

**Solution structure of tRNA**

Data from numerous investigations of the solution structure of tRNA generally agrees with the crystal structure of yeast tRNA^{Phe}. These studies include investigation of the susceptibility of tRNAs to a variety of nucleases (for review see Rich and RajBhandary, 1976), the exchange of tritiated water with H-8 of purines (reviewed by Schimmel and Redfield, 1980), binding of complementary oligonucleotides to tRNAs to probe for single-stranded regions (Uhlenbeck, 1972; Freier and Tinoco, 1975), and perhaps most importantly chemical modifications of tRNA (reviewed by Holbrook and Kim, 1983). A recent study comparing the reactivity of yeast
tRNA$^{\text{Asp}}$ and tRNA$^{\text{Phe}}$ to ethynitrosourea with the crystal structures of both tRNAs, again supports the contention that these molecules have similar structures in the crystal and in solution (Romby et al., 1985). In addition, a number of physical studies of tRNA in solution such as small-angle X-ray scattering (Nilsson et al., 1982), laser Raman spectroscopy (Chen et al., 1975), and NMR spectroscopy (reviewed by Reid, 1981) have confirmed that in general, the solution structure of transfer RNA closely approximates that of the crystal structure of yeast tRNA$^{\text{Phe}}$.

**Dynamics of tRNA**

The three-dimensional structure determined by X-ray crystallographic methods is a static structure representing the average of a population of dynamically fluctuating structures. When tRNA interacts with other macromolecules, conformational changes in the tRNA structure are generally assumed to take place. A complete understanding of transfer RNA will, therefore, involve knowledge of conformational variations and dynamics of this molecule alone, as well as in association with other relevant biomacromolecules.

**Conformational states in solution**

The ability of transfer RNA to undergo conformational changes in solution has been studied intensively (for review see Crothers and Cole, 1978 or Rigler and Wintermeyer, 1983). The early studies of Crothers and his coworkers established the importance of temperature and counterions in determining the conformation of tRNA (Cole et al., 1972). More recently, fluorescence studies using tRNA labeled with ethidium in the D loop or in the anticodon loop have distinguished three conformational states (T1, T2, T3); the distribution of these states is dependent on Mg$^{++}$, spermine, and temperature (Ehrenberg et al., 1979, Nilsson et al., 1983). T3 is the
predominant state at high concentrations of Mg$^{++}$ and/or spermine, and it was concluded that tRNA in this state may have a structure similar to the crystal structure of yeast tRNA$^{\text{Phe}}$.

Further evidence for magnesium and temperature induced conformational changes in tRNA has been provided by $^{31}$P NMR studies (Salemink et al., 1980; Gorenstein and Goldfield, 1982). Also, proton and $^{13}$C NMR studies have provided evidence for two different conformations of the T loop in tRNAs in solution (Kastrup and Schmidt, 1975, 1978; Kopper et al., 1983). Several other studies have shown that mildly acidic pH can induce conformational transitions in tRNA (Bina-Stein and Crothers, 1975; Steinmetz-Kayne et al., 1977).

The biological significance of tRNA conformational changes is not well understood, and questions still remain regarding the role of conformational changes of tRNA in protein synthesis. For example, aminoacyl-tRNA is distinguished from nonaminoacylated tRNA in a number of biological processes, including interaction with EF-Tu and ribosome binding, leading to speculation that tRNA molecules undergo a conformational change upon aminoacylation. However, the effect of aminoacylation on the structure of tRNA has been a controversial issue. Some evidence suggests that aminoacylation causes conformational changes in the tRNA molecule resulting in a "more extended" conformation (Potts et al., 1981). Other studies, such as NMR analysis of aminoacylated tRNA, have found little or no structural alterations upon aminoacylation (Wong et al., 1973; Davanloo et al., 1979). Also, analysis of crystals of N-acetyl-Phe-tRNA$^{\text{Phe}}$ has revealed no significant differences from tRNA$^{\text{Phe}}$, although small changes could not be ruled out (Rich et al., 1980). It seems that if any changes in tRNA conformation occur on aminoacylation, they are quite small.

Although several conformational changes have been proposed to occur in tRNA upon codon binding (discussed below), conclusive evidence about
the existence of these changes and their role in protein synthesis is not yet available.

Codon-Anticodon Interaction

Recognition of a codon triplet on messenger RNA by the appropriate transfer RNA is one of the most important steps in protein biosynthesis. It is, in part, the specificity of this interaction that ensures the proper sequence of amino acids in the growing peptide chain. Due to this importance, the codon-anticodon interaction has been studied extensively. A complete understanding of this process will require detailed knowledge of the structure and dynamics of codon-anticodon complexes.

Interaction of the anticodon loop of tRNAs with oligonucleotide codons has been studied by equilibrium dialysis (Uhlenbeck et al., 1970; Eisinger and Spahr, 1973; Freier and Tinoco, 1975), fluorescence measurements (Yoon et al., 1975), and recently by $^1$H NMR (Geerdes et al., 1980a, 1980b; Clore et al., 1984). Association constants for codon-anticodon complexes are significantly higher than those for the association of complementary tri- or tetranucleotides (Jaskunas et al., 1968), implying that some feature of the anticodon loop is involved in stabilizing the codon-anticodon interaction (Yoon et al., 1975). This concept is further supported by the finding that two tRNA molecules with complementary anticodons can form a complex whose association constant is several orders of magnitude greater than that for binding of complementary oligonucleotides (Eisinger, 1971; Grosjean et al., 1976). The ideal base stacking of the anticodon in the crystal structure of tRNA$^{\text{Phe}}$ (Quigley and Rich, 1976; also see previous section on tRNA$^{\text{Phe}}$ crystal structure) provides some clue to the nature of this improved stability, and several studies have shown that the presence of modified bases adjacent to the anticodon also stabilizes the interaction with codon (Pongs and Reinwald,
Conformational changes

Much conflicting evidence has been presented concerning the possibility of codon-induced conformational changes in tRNA and their role in protein synthesis. Two types of structural transitions have been proposed: one in the anticodon loop itself, and the other some distance from the anticodon, involving disruption of the tertiary interactions between the T and D loops.

The 3'-stacked conformation of the anticodon loop seen in the crystal structures of several tRNAs was first proposed by Fuller and Hodgson (1967). Since then, several models of protein synthesis have proposed a transition between the 3'-stacked structure and a conformation with the anticodon stacked on the 5' side of the loop (Woese, 1970; Crick, 1976; Lake; 1977).

Considerable evidence suggests that the structure of the anticodon loop is flexible and very sensitive to solution conditions, even more labile than the tertiary structure of the molecule. The thermal vibrational motions of the nucleotides in the tRNA^Phe crystal structure indicate that the anticodon loop is among the most flexible parts of the molecule (Kim, 1980). Conformational flexibility in this loop has also been shown by NMR (Salemink et al., 1980; Gorenstein and Goldfield, 1982). These studies show that at high [Mg^{2+}] the anticodon loop is present in two different conformations, and under all conditions, anticodon loop structure is very temperature sensitive. A number of fluorescence studies involving the Y base, 3'-adjacent to the anticodon in yeast tRNA^Phe also indicate that the anticodon loop may be present in at least two conformations (Ehrenberg et al., 1979; Wells, 1984). Urbake and Maass (1978) have suggested that a relaxation process observed in temperature jump fluores-
cence studies of tRNA^{Phe} is the result of a conversion between the 3'- and 5'-stacked conformations. Later studies by Labuda and Porshke (1980) indicate that the codon UUC binds preferentially to one of the two anticodon conformations.

Results of oligonucleotide binding experiments also indicate that the anticodon may be stacked on the 5'-side of the loop. In the 3'-stacked structure, a sharp U-turn separates the anticodon triplet from the invariant U-33 located 5' to the anticodon. In this conformation, U-33 is not available for base-pairing with oligonucleotides that are complementary to the anticodon and the adjacent 5'-sequence. However, several studies show that oligonucleotides which are complementary to U-33 in addition to the anticodon, bind significantly more tightly than the corresponding codon triplets (Uhlenbeck et al., 1970; Eisinger and Spahr, 1973; Yoon et al., 1975; Geerdes et al., 1980a). The higher affinity of these tetranucleotides for the anticodon of a tRNA suggests additional base-pairing between the 3'-terminal adenosine and U-33. An alternative explanation for the increased affinity is that the fourth base of the tetranucleotide (A), rather than base-pairing with U-33, stabilizes the oligonucleotide binding by stacking on the preceding base-pair (3'-dangling end effect) (Westhof et al., 1983).

The role of a conversion between the 3'- and 5'-stacked conformation of the anticodon in protein synthesis has been challenged in recent years. A number of studies, including detailed analysis of fluorophore-derivatized tRNA^{Phe} (Odom et al., 1978; Paulsen et al., 1982), chemical modification (Douthwaite et al., 1983), and photo-cross-linking between the tRNA anticodon and mRNA (Matzke et al., 1980; Steiner et al., 1984; Ciesiolka et al., 1985) suggest that the anticodon loop retains the conformation seen in crystals, in solution, upon binding to ribosomes, and during the translocation step of protein synthesis.
Conformational changes in distant parts of the tRNA molecule have been attributed to the association of codon with the anticodon, and in particular it has been proposed that the interactions between the T and D loops are disrupted as a result of this recognition process (Möller et al., 1980). This structural rearrangement has been suggested to make the conserved TyCG sequence in the T loop available for interaction with a complementary sequence in one of several ribosomal RNAs. 5S rRNA was first implicated as the site of interaction (Forget and Weissman, 1967; Schwarz et al., 1976) but this hypothesis was later discounted by investigations with 5S RNA lacking the complementary binding site (Pace et al., 1982; Zagorska et al., 1984). Recent studies suggest that 16S rRNA has the potential to bind the GTyCG sequence of an elongator tRNA (Hilk and Sprinzl, 1985).

In the crystal structure of yeast tRNA^{Phe} the T loop interacts with the D loop, forming a number of tertiary base-pairs rendering the TyCG sequence unavailable for pairing to complementary oligonucleotides. And in solution, neither yeast tRNA^{Phe} (Pongs et al., 1973) nor tRNA^{Met}_{f} (Freier and Tinoco, 1975) bind C_{p}G_{p}A_{p}A. Significant binding of C_{p}G_{p}A_{p}A to yeast tRNA^{Phe} was shown to occur when (U_{p})_{8} is present (Schwarz et al., 1976), which suggests that codon binding causes a conformational change in the tRNA that disrupts the interaction between the T and D loops, making the TyCG sequence available. The three-dimensional structure of yeast tRNA^{Asp} molecules associated through their anticodons reveals a destabilization of the interactions between the D and T loops, and Westhof et al. (1985) propose that this is a consequence of the anticodon-anticodon self-pairing (Westhof et al., 1985).

Other studies using ^{1}H NMR do not detect any gross conformational change in the structure of tRNA^{Phe} as a result of codon binding (Geerdes et al., 1978). The possibility of codon-induced rearrangement of the T-D
loop interactions and its role in protein synthesis remains controversial.

Codon binding has also been shown to induce association of transfer RNA molecules, resulting in the formation of dimers (Porschke and Labuda, 1982, 1983; Geerdes et al., 1980b) or a complex of 9±2 RNA molecules (Clore et al., 1984). This effect appears to be specific for trinucleotide codons. In one case U_pU_pC binding to yeast tRNA^{Phe} resulted in dimer formation whereas the tetranucleotide U_pU_pC_pA did not induce association (Geerdes et al., 1980b).

NMR Studies of tRNA

High resolution nuclear magnetic resonance (NMR) is currently the most informative spectroscopic tool available to probe the solution conformation and dynamics of tRNA. The particular advantage of NMR is that signals can be obtained from nuclei in many parts of the molecule simultaneously, and several types of information can be obtained from each reporter. Before detailed structural and biochemical information can be obtained from NMR studies, resonances in the spectra must be assigned to specific nuclei in the molecule. Much of the recent work has been concerned with making such identifications, and these studies are opening the way for more detailed study of tRNA in solution and of its interaction with biologically relevant molecules.

Two naturally occurring isotopes in RNA, ^1H and ^31P, are amenable to NMR studies, and isotope enrichment techniques are being used to study other nuclei such as ^13C and ^15N. The majority of NMR studies of tRNA involve proton NMR; these investigations have centered on the hydrogen-bonded imino protons (Reid, 1981), and the modified bases in tRNA (Kastrup and Schmidt, 1978). Changes in the phosphodiester backbone of tRNA can be monitored using ^31P NMR (Salemink et al., 1980; Gorenstein and Goldfield,
1982), and $^{13}$C NMR has been used to study both the methylated bases
(Kopper et al., 1983; Smith et al., 1985) and the major constituents of
tRNA (Schweizer et al., 1984; Schmidt et al., 1983) Recently, $^{15}$N NMR has
been used to study the conformation of pseudouridine in several tRNAs
(Griffey et al., 1985). A brief description of several applications of
NMR to study tRNA follows. Several excellent recent reviews may be
consulted for more detailed information on this subject (Schimmel and
Redfield, 1980; Reid, 1981; Rigler and Wintermeyer; 1983).

**Assignment of proton resonances**

Major advances in assignment of the imino protons in low-field NMR
spectra of tRNA have been made recently using nuclear Overhauser effect
(NOE) experiments (Reid, 1981; Roy and Redfield, 1983; (Hyde and Reid,
1985). Two dimensional NOE experiments have even resulted in the complete
assignment of the imino proton resonances in the spectra of phenylalanine
tRNA from yeast (Hilbers et al., 1983) and *E. coli* tRNA$^{Val}$ (Hare et al.,
1985). Assignment of the high-field methyl spectra has been accomplished
by comparing the NMR spectra of tRNA at high temperature with that of
model methylated nucleosides. The assigned peaks are then traced back to
their native chemical shifts by lowering the temperature. With reliable
assignments the interaction of tRNA with other molecules such as cations,
codons, and enzymes can be studied and interpreted using proton NMR.

**Melting**

Thermal denaturation of tRNA has been the subject of several NMR
studies. Kastrup and Schmidt (1975, 1978) monitored the sequential
thermal unfolding of tRNA$^{Val}$ using $^1$H NMR of modified bases. Three separate structural transitions are observed as the temperature is increased: disruption of tertiary structure, changes in the anticodon stem, and finally melting of the TyC helix. Evidence also supports the existence of
multiple conformations of the ribothymidine residue at temperatures below the $T_m$ (Kastrup and Schmidt, 1978).

The thermal unfolding sequence of tRNA$^{\text{Phe}}$ has recently been studied using imino proton exchange (Johnson and Redfield, 1981; Roy and Redfield, 1983). In the absence of magnesium, acceptor stem and tertiary resonances melt almost concurrently followed by the dihydrouridine stem; in high [Mg$^{++}$] the D stem becomes the most stable helical structure. At low temperature, the acceptor stem shows increased lability at both ends, indicating that individual stems do not necessarily melt in a concerted fashion. These studies also show that tertiary interactions in tRNA are not always the most labile as previously assumed. Similar observations have been made with E. coli tRNA$^{\text{LYS}}$ by Reid (1981) where the anticodon helix melts before unfolding of tertiary structure occurs.

**Ion binding to tRNA**

Addition of magnesium to tRNA causes pronounced shifting of some low-field resonances in $^1$H NMR spectra (Reid, 1981). Recent studies by Hyde and Reid (1985) with E. coli tRNA$^{\text{Phe}}$ show effects of Mg$^{++}$ on resonances from all stems of the molecule, but different effects are observed for different peaks. The tightest binding site observed is near U12-A23. These studies also show that at low [Mg$^{++}$] tRNA$^{\text{Phe}}$ exists as a mixture of two conformations in slow exchange, differing at positions 12 and 13 in the D stem.

The binding of Mn$^{++}$ to tRNA has been studied via its paramagnetic relaxation effects. This ion binds in the hinge region between the D and T loops of tRNA$^{\text{Phe}}$, as determined crystallographically (Jack et al., 1977; Quigley et al., 1978). Chao and Kearns (1977) have interpreted selective broadening of proton NMR signals by Mn$^{++}$ in the spectrum of yeast tRNA$^{\text{Phe}}$ to reflect sequential Mn$^{++}$ binding at several sites in the order $^4$U8-A14,
Hurd et al., (1979) investigated several tRNAs at lower Mn\textsuperscript{2+} levels and found a single site close to the s\textsuperscript{4}U8-A14 tertiary base-pair. \( ^{13}C \) NMR studies of [4-\textsuperscript{13}C] uracil labeled tRNA\textsubscript{Val} show that the dihydrouridine residue at position 17 was affected by Mn\textsuperscript{2+} before the s\textsuperscript{4}U-8 peak (Schweizer, 1980).

Contrasting the previously mentioned studies, which indicate discrete cation binding sites, \( ^{31}p \) NMR studies by Gueron and Leroy (1982) indicate very similar Mn\textsuperscript{2+} lifetimes on all phosphates with no specific tight binding sites.

Spermine binding to \( E.\ ooll \) tRNA\textsubscript{Phe} has been reported to mainly affect \( ^{1}H \) NMR resonances from base pairs G15-C48 and s\textsuperscript{4}U8-A14 (Hyde and Reid, 1985), whereas Gorenstein and Goldfield (1982) found a major effect of added spermine on the \( ^{31}p \) resonances from anticodon residues in yeast tRNA\textsubscript{Phe}. Two bound spermine molecules are found in the X-ray structure of tRNA\textsubscript{Phe}, one in the anticodon helix and a second near the D stem, (Quigley et al., 1978, see Figure 1B).

### Interaction with proteins

One ultimate goal of NMR studies of tRNA is analysis of tRNA recognition by specific enzymes. Schulman and co-workers (1975) investigated the effects on the \( ^{1}H \) NMR spectrum of \( E.\ ooll \) tRNA\textsubscript{Glu} of binding Glutamyl-tRNA synthetase and the elongation factor EF-Tu. Conducted at 25\textdegree C, these studies produced very broad unresolved spectra upon complex formation with either protein. The authors concluded, however, that the tRNA does not change its secondary structure upon binding to either protein since no base-pairs were broken.

More recently, advantage has been taken of thermophilic synthetase enzymes that are stable up to 65\textdegree C. Schweizer and co-workers (1984) studied the cognate complex between [4-\textsuperscript{13}C] uracil-labeled \( E.\ ooll \) tRNA\textsubscript{Val}
and *B. stearothermophilus* valyl-tRNA synthetase (VRS). The greatest effects of this interaction were on $^{13}$C peaks assigned to $s^4$UB, rT54, and V34, suggesting that VRS is in contact with the anticodon, D, and T loops.

Incorporation of 5-Fluorouracil into Transfer RNA

The pyrimidine analog 5-fluorouracil (FUra) was first synthesized in 1957, primarily as a potential drug for the treatment of cancer (Duschinsky et al., 1957). The primary mechanism of the anticancer activity of FUra is generally believed to involve inhibition of thymidylate synthetase by the *in vivo* 5-fluorouracil metabolite, 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) (Myers, 1981). FdUMP acts as a suicide inhibitor of thymidylate synthetase, giving rise to a stable structure resembling a transient intermediate in the normal enzyme reaction (Abeles and Maycock, 1980). This interaction has been of great value in studying the mechanism of thymidylate synthetase activity.

Under certain conditions FdUTP is formed and can be incorporated into DNA (Major et al., 1982). Also, in many systems FUra is converted to 5-fluorouridine 5'triphosphate and incorporated into RNA (Horowitz and Chargaff, 1959). The complex metabolism and biochemical mechanisms of 5-fluorouracil action were recently reviewed by Heildelberger et al. (1983).

5-Fluorouracil is incorporated into bacterial transfer RNAs (Horowitz and Chargaff, 1959) as a substitute for uridine and uridine-derived minor nucleosides (Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1972). Under certain conditions this replacement can be essentially complete resulting in substituted tRNAs that lack modified nucleosides such as pseudouridine, ribothymidine, 5,6-dihydouridine, and 4-thiouridine (Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1969). Studies of FUra-substituted transfer RNA have been conducted to investi-
gate the effects of FUra replacement on the structure and biological functions of these molecules and to study the role of modified nucleosides in transfer RNA activity.

Unfractionated FUra-containing tRNA from *E. coli* is able to accept all amino acids (Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1969). However, several (FUra)tRNAs are aminoacylated at significantly lower rates and to lower extents than their normal counterparts (Ramberg et al., 1978). Lysine tRNA is the most inhibited, being only 6% as active as normal tRNA^lys^*.FUra-substituted Lys-tRNA^lys^ is also impaired in its ability to bind to ribosomes and function in polypeptide synthesis (Ramberg et al., 1978). Aminoacylation with aspartate, glutamate, glutamine, and histidine is also inhibited. All these analogue-substituted tRNAs with reduced activity contain a 5-fluorouridine residue in the second position of their anticodons, and this substitution may be, at least in part, responsible for their lowered activities (Ramberg et al., 1978).

With the few exceptions noted above, FUra-substituted tRNAs from *E. coli* appear to be fully functional in protein synthesis. Examined in detail, the properties of purified FUra-substituted valine tRNA have been found to differ little from normal tRNA^Val^ (Ofengand et al., 1974). (FUra)tRNA^Val^ is aminoacylated with similar kinetics as normal tRNA^Val^ (Horowitz et al., 1974). Ternary complex formation with EF-Tu-GTP, nonenzymatic binding to the ribosomal P-site, EF-Tu-dependent binding to the ribosomal A-site, and polypeptide synthesis all occur normally with FUra-substituted tRNA^Val^ (Ofengand et al., 1974). These studies clearly demonstrate that in every biological function tested, FUra-substituted tRNA^Val^ retains full activity. Furthermore, FUra-containing tRNA is fully functional in the tRNA-dependent synthesis of pppGpp in the stringent response (Chinali et al., 1978). (FUra)tRNA^Met^ has also been found to be fully
active with regard to aminoacylation and formylation (Hills et al., 1983).

Incorporation of FUra also has little effect on the physical properties of tRNA_val, including the temperature-absorbance melting profile, circular dichroism spectra, and electrophoretic mobility in polyacrylamide gels (Horowitz et al., 1974). FUra-substituted tRNA_val, therefore, represents an ideal system for studies of transfer RNA structure and function by 19F NMR. Most of the studies presented here involve this tRNA.

**19F NMR Studies of FUra-Substituted tRNA**

19F NMR provides a powerful probe of the solution structure and dynamics of FUra-substituted tRNA. Advantages of using fluorine-19 in NMR spectroscopy include: high sensitivity of detection, 100% natural abundance, a large range of 19F chemical shifts, and extreme sensitivity of 19F chemical shifts to the environment of the nucleus. Also, 19F NMR spectroscopy of fluorinated tRNAs is ideally suited for investigation of tRNA-protein interactions without interference from protein signals.

Prior to this study, 19F NMR spectra of two purified FUra-substituted E. coli tRNAs, tRNA_val (Horowitz et al., 1977) and tRNA_met (Hills et al., 1983) had been obtained. Cloverleaf structures of these analogue substituted tRNAs, along with FUra-substituted tRNA_met, (purified in this study), are shown in Figure 2. FUra residues occur in virtually every stem and loop of these tRNAs, and each can serve as a reporter of structural changes in its vicinity.

Representative 282 MHz 19F NMR spectra of (FUra)tRNA_val and (FUra)tRNA_met are shown in Figure 3A and 3B, respectively; also indicated are the relative areas of each region of the spectra, obtained by integration on the spectrometer, and the peak designations. Spectra of (FUra)tRNA_val (Fig. 3A) show 11-12 resolved resonances for the 14 incorpo-
Figure 2. Nucleotide sequence (cloverleaf structure) of *E. coli* tRNA\textsubscript{Val}, tRNA\textsubscript{Met}, and tRNA\textsubscript{Met} with uracil and uracil-derived nucleosides replaced by 5-fluorouracil (F). The bases substituted by FUra which are invariant in all tRNAs (see Fig. 1A) are enclosed in boxes. FUra residues involved in tertiary hydrogen bonds with other nucleosides, based on the crystal structure of yeast tRNA\textsuperscript{Phe} (see Fig. 1B), are connected by solid lines.
Figure 3. $^{19}$F NMR spectra of 5-fluorouracil-substituted tRNA$^{Val}_1$ (A) and tRNA$^{Met}_f$ (B). The spectra were recorded at 282 MHz at 25°C with tRNA in 50 mM sodium cacodylate buffer, pH 6.0, containing 15 mM MgCl$_2$, 10 mM NaCl, 1 mM EDTA, and 10% $^2$H$_2$O as an internal lock. Chemical shifts are given in ppm from 5-fluorouracil. Areas obtained by integration on the spectrometer, and peak designations are indicated.
A

(i)

(ii)

CHEMICAL SHIFT (PPM FROM FUrA)

B

(i)

(ii)

CHEMICAL SHIFT (PPM FROM FUrA)
rated FUra residues in the molecule (Fig. 2), and 9-10 resolved peaks are seen in spectra of (FUra)tRNA_{Met}^f (Fig. 3B) from the 12 FUra residues in this fluorinated tRNA (Fig. 2).

The $^{19}$F resonances in each spectrum are distributed over a large range of chemical shift positions, from ca. 0.5 to 8.0 ppm downfield from the standard free FUra (= 0 ppm). Comparison of the two spectra (Fig. 3A and 3B) shows that while there are similarities, each tRNA yields a unique characteristic spectrum. One notable similarity between the spectra is that in each case the peaks can be grouped into three categories based on chemical shift position. Each spectrum contains a central cluster of resonances between ca. 3.8 and 5.0 ppm, several resolved resonances located downfield from 5 ppm, and a number of peaks upfield from the central cluster. Evidence has been presented (Horowitz et al., 1977; Hardin, 1984; Hardin et al., 1986) supporting the hypothesis that these groupings correspond to FUra residues in three distinct structural environments in the tRNA molecule: hydrogen-bonded bases in helical domains (upfield), non-hydrogen-bonded bases in loops (central), and FUra residues participating in tertiary interactions (downfield).

FUra-containing tRNA_{Met}^f and tRNA_{Val}^Val can be separated into two isoaccepting species termed the A and B forms (Hills et al., 1983; Hardin, 1984). $^{19}$F NMR spectra of the two forms differ by the shift of one $^{19}$F peak from ca. 4.5 to 4.8 ppm in isoacceptor B, upfield to -15 ppm in isoacceptor A. Further studies have demonstrated that the peak at ca. -15 ppm is due to a ring-opened fluorodihydrouridine substituent in the A form of these tRNAs (Horowitz et al., 1983). Apparently, the enzyme that normally reduces uridine to dihydrouridine is capable of recognizing 5-fluorouracil to some extent, and reduces it to fluorodihydrouridine. This is the only known example of enzymatic recognition and modification of 5-fluorouridine in tRNA.
In the A form of (FUra)\textsubscript{tRNA\textsuperscript{Met}}, the modified FUra was shown to be located at position 20, normally occupied by 5,6-dihydrouridine (Hills et al., 1983). This finding allowed assignment of a resonance at 4.8 ppm in the \textsuperscript{19}F NMR spectrum of (FUra)\textsubscript{tRNA\textsuperscript{Met}} (B) to FUra-20 (Hills et al., 1983). It has been suggested that a similar situation exists in (FUra)\textsubscript{tRNA\textsuperscript{Val}}, with the modified FUra in the A form being located at the site of the only dihydrouracil in the molecule (position 17), but conclusive evidence was not available (Hardin, 1984).

Several other preliminary peak assignments in the \textsuperscript{19}F NMR spectrum of (FUra)\textsubscript{tRNA\textsuperscript{Val}} have been made (Hardin, 1984). However, most of the resonances remain unassigned, and need to be assigned to allow interpretation of spectral changes at the molecular level.

In this study, FUra-substituted tRNA\textsubscript{Met} was purified, its \textsuperscript{19}F NMR spectrum obtained and then compared to those of (FUra)\textsubscript{tRNA\textsuperscript{Met}} and (FUra)\textsubscript{tRNA\textsuperscript{Val}}. The effects of pH, ion binding, and temperature on the \textsuperscript{19}F NMR spectra of FUra-substituted tRNA were also examined. Reaction of FUra-containing tRNA with bisulfite and psoralens was investigated as a means for assigning peaks in the spectra. Finally, codon-anticodon interaction in FUra-substituted tRNA was examined in detail by \textsuperscript{19}F NMR spectroscopy.
EXPERIMENTAL PROCEDURES

Materials

5-fluorouracil was donated by Hoffmann-LaRoche, and calcium leucovorin was a gift from Lederle Laboratories. *Escherichia coli* strain B was supplied by Grain Processing Corporation, Muscatine Iowa. Sephacryl S-200, Sepharose 4B, DEAE-Sephadex A-50 and A-25 were all purchased from Pharmacia. BD-cellulose and Bio-Gel P2 were supplied by Bio-Rad Laboratories, DEAE-cellulose (DE-32) was a Whatman product, and cellulose thin-layer sheets (5502) were from EM Laboratories. RPC-5 adsorbant was prepared according to Method C of Pearson et al. (1971) using Adogen 464 (Ashland Chemical Co.) and Plascon CTFE 2300 powder (Allied Chemical Co.), which were gifts of Dr. Ivan Kaiser, Dept of Biochemistry, University of Wyoming, Laramie WY. Adenosine 5'-[γ-32P] triphosphate was purchased from New England Nuclear. Radioactive amino acids were obtained from New England Nuclear or ICN, and 3H-labeled KBH₄ was from Amersham Corp. Spermine tetrahydrochloride was purchased from Calbiochem Inc. Ribonuclease H was a product of Bethesda Research Laboratories. Polynucleotide phosphorylase, calf intestinal phosphatase (Molecular Biology Grade), and G₃U were from Boehringer Mannheim. Ribonuclease T₁, Ribonuclease A, Pyrophosporyl chloride, Pyronin Y, A₃P₃A₃, U₃P₃A₃, and C₃G and ribonucleosides were all obtained from Sigma. *E. coli* tRNA¹⁰ with a specific activity of 1450 pmol/A₂₆₀ was from Subriden RNA, Bacterial alkaline phosphatase (BAPF) and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp. T₄ RNA ligase and polynucleotide kinase were products of P and L Pharmacia Biochemicals. Eastman-Kodak X-Omat XAR-5 film was used for fluorography and autoradiography. The scintillators 2,5-diphenyloxazol (PPO) and 1,4-bis-[2-(5-phenyloxazol)]-benzene (POPOP) were Fischer products as were acrylamide and N,N-methylene bisacrylamide which were recrystallized from acetone prior to use. Sodium cacodylate and
deuterium oxide (99.8%) were from Aldrich. All other reagents were of analytical grade or better.

The 10-formyltetrahydrofolate used in formylation assays was prepared by the method Dubnoff and Maitra (1971). Calcium leucovorin (5-formyltetrahydrofolate) was converted to 5,10-methyltetrahydrofolate by incubation at room temperature, in the dark, with 0.1 M HCl for 4 h. This product was stored at -20°C and converted to 10-formyltetrahydrofolate by treatment with 40 mM Tris-HCl, pH 8, and 80 mM KOH just prior to use.

3',5'-Nucleoside bisphosphates used in RNA ligase catalyzed synthesis of oligonucleotides were prepared from the corresponding nucleosides and pyrophosphoryl chloride by the method described by Barrio et al., (1978). A mixture of nucleoside (0.15 mmol) and pyrophosphoryl chloride (1.5 mmol) was stirred at -10°C in an ethanol/ice bath. After 4 h, the reaction was quenched by addition of ice and 5-10 mL of 0.5 M TEAB, pH 8.0. The solution was evaporated to dryness under vacuum at 30°C and the residue was then dissolved in 50% methanol and evaporated to dryness. This step was repeated several times to remove all remaining TEAB. The resulting bisphosphates were purified by chromatography on a column of DEAE-cellulose (2.3 x 36 cm) using a linear gradient of 50 to 400 mM TEAB, pH 8.0. Yields were 50-90% of an unresolved mixture of nucleoside 2',5'- and 3',5'-bisphosphates, as judged by 31P NMR. This mixture was used directly in ligation reactions because the 2',5'-bisphosphates do not serve as either substrates or as competitive inhibitors (England and Uhlenbeck, 1978).

All glassware was baked at 140°C for 6 h or longer and water was autoclaved to minimize nuclease activity. Phenol was distilled prior to use to remove impurities, and then stored at 4°C in the dark. Dialysis membranes were from Bethesda Research Laboratories and had been treated to remove heavy metal contaminants. The membranes were stored in 25% ethanol.
with 1 mM sodium azide and were washed thoroughly with autoclaved water prior to use. Transfer RNA concentrations were determined from spectrophotometric measurements at 260 nm assuming a value of $E_{260}^{1%} = 24$.

Methods

Growth of *E. coli* cells in the presence of 5-fluorouracil

*Escherichia coli* strain B was grown at 37°C in a minimal glucose-salts medium (Demerec and Cahn, 1953) in 15 L glass carboys with aeration. When the cells reached exponential growth, 5-fluorouracil and thymidine were added at a final concentration of 25 mg/L each. After incubation for 3 hr, the cells were concentrated with an Amicon Pellicon system and collected by centrifugation for 10 min at 4000 x g in a Beckman J2-21 centrifuge (JA-10 rotor). Cells were then washed twice with 10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate and stored at -20°C. Yields were 20-35 g of cell paste/15 L culture.

tRNA preparation

Unfractionated RNA was prepared from FUra-treated bacteria by phenol extraction of whole cells as described by Zubay (1966). After the phenol treatment, the RNA was precipitated from the aqueous phase by addition of 0.1 volume of 20% potassium acetate, pH 5.0 and two volumes of ethanol. The precipitated RNA was stored at -20°C at least 1 h, collected by centrifugation at 10,000 x g for 10 min, and then dissolved in 300 mM sodium acetate, pH 7.0, at a concentration of 2.5 mg/mL. High molecular weight RNA (16S and 23S rRNA) was removed from the preparation by precipitation with isopropyl alcohol (0.54 volumes) at 20°C (Zubay, 1966). Contaminating 5S rRNA was removed from the tRNA by gel filtration on Sephacryl S-200 (2.5 x 235 cm) at room temperature, using 0.5 M NaCl in 0.14 M sodium acetate buffer, pH 4.5, as eluant (Horowitz et al., 1974).
Purification of FUra-substituted tRNA$_{\text{Met}}$

5-fluorouracil-substituted tRNA$_{\text{Met}}$ was purified using minor modifications of the procedure described by Hills et al. (1983) for the purification of FUra-substituted initiator methionine tRNA. This method involves a series of column chromatography steps. The methionine tRNA-containing fractions from each column were located by aminoacylation assay using $^3$H-L-methionine, pooled, and the RNA collected by precipitation with ethanol. The specific activity of the tRNA from each column was determined by assaying for acceptance of $^{14}$C-L-methionine.

FUra-containing tRNA was separated from unsubstituted tRNA by ion exchange chromatography on DEAE-cellulose (5 x 750 cm) at pH 8.9 (Kaiser, 1969). The column was developed at room temperature with a concave NaCl gradient from 0.325 M (6 L) to 0.6 M (2 L) in 20 mM Tris-HCl, pH 8.9. Fractions containing methionine tRNA were pooled and assayed for the ability to be formylated, in order to distinguish between tRNA$_{\text{f}}$ and initiator tRNA$_{\text{G}}$.

FUra-substituted tRNA$_{\text{m}}$ was then further fractionated by chromatography at 5°C on a Sepharose 4B column (2 x 94 cm) using a reverse ammonium sulfate gradient from 1.5 M to 0.0 M (NH$_4$)$_2$SO$_4$ (Holmes et al., 1975; Colantuoni et al. 1978). The pooled tRNA$_{\text{Met}}$-containing fractions were desalted on DEAE-cellulose as described by Gillam and Tener (1980), and the RNA was then further purified on DEAE-Sephadex A-50 (1.5 x 150 cm) at room temperature using a 2 L linear gradient from 0.425 M to 0.500 M NaCl in 20 mM Tris-HCl, pH 7.5, and 8 mM MgCl$_2$ (Nishimura, 1971). A final purification step involved chromatography at 5°C on benzoylated DEAE-cellulose (0.9 x 125 cm) using a 500 mL linear gradient from 0.4 to 1.0 M NaCl in 10 mM sodium acetate, pH 4.5, and 10 mM MgCl$_2$. This step separated two isoaccepting forms of the FUra-substituted tRNA$_{\text{Met}}$. Only a minimal further purification of either form of the tRNA was achieved by
reverse phase chromatography on RPC-5 (Pearson et al., 1971) using a 0.9 x 66 cm column developed with a 500 mL linear gradient from 0.5 M to 1.0 M NaCl in 10 mM sodium acetate, pH 4.5, and 10 mM MgCl₂.

**Purification of FUrA-substituted tRNA\textsuperscript{Met}**

Purified FUrA-containing initiator methionine tRNA was prepared as previously described (Hills et al., 1983), using techniques similar to those outlined for the purification of (FUrA)tRNA\textsuperscript{Met}. Briefly, the procedure involved chromatography on DEAE-cellulose, Sepharose 4B, and DEAE-Sephadex A-50. BD-cellulose was used to separate the A and B forms of FUrA-substituted tRNA\textsuperscript{Met}. Both isoacceptors had specific activities of over 1400 pmol/A\textsubscript{260}, and were formylated to the extent of 111% (form-A) and 92% (form B).

**Purification of FUrA-substituted tRNA\textsuperscript{Val}**

tRNA\textsuperscript{Val} was purified from 5-fluorouracil treated E. coli by minor modifications of the procedure described previously for purification of FUrA-containing methionine tRNAs. In outline, the method involves DEAE-cellulose chromatography at pH 8.9, followed by chromatography on Sepharose 4B and finally BD-cellulose. The purified tRNA accepted over 1400 pmol \textsuperscript{14}C-L-valine per A\textsubscript{260} unit. FUrA-substituted tRNA\textsuperscript{Val} was resolved into two isoaccepting forms, (A) and (B), on an RPC-5 reverse phase chromatography column (Pearson et al., 1971), as described by Hardin (1984). Unless the two forms were required separately, a mixture containing mostly form B, obtained after BD-cellulose chromatography, was used in these studies.

**Aminoacylation assay**

Methionine or valine acceptance activity of tRNA samples was measured in 100 µL reactions containing 100 mM HEPES, pH 7.5, 20 mM magnesium
acetate, 10 mM KCl, 10 mM ATP, 1 mM dithiothreitol, 300 μg/mL partially purified aminoacyl-tRNA-synthetases (Meunch and Berg, 1966), 60-80 μM radiolabeled amino acid (either $^{14}$C-labeled, specific activity 50 mCi/mmol or $^{3}$H-labeled, specific activity 0.2-1.0 Ci/mmol), and varying amounts of tRNA. Reactions were incubated 30 min at 37°C, and stopped by addition of ice cold 5% trichloroacetic acid. The mixtures were incubated on ice for 10 min, the precipitates were collected on Millipore filters (0.45 μm pore size), washed twice with cold 5% trichloroacetic acid, dried and counted in a Beckman LS 100 or LS 7500 liquid scintillation counter, using a toluene based scintillation cocktail containing 0.5% PPO and 0.01% POPOP.

**Formylation assay**

Assays for the formylation of methionyl-tRNA were carried out by aminoacylating the tRNA in the presence of the formyl donor 10-formyl-tetrahydrofolate and treating a portion of the reaction with Cu$^{++}$ at pH 5.5. Under these conditions, unformylated methionyl-tRNA is hydrolyzed, but formylmethionyl-tRNA$^{Met}$ is stable (Schofield and Zamecnik 1968). Transfer RNA samples were aminoacylated as described previously for aminoacylation assay in a 500 μL reaction mixture containing 600 μM 10-formyl-tetrahydrofolate. Sufficient transformylase was present in the partially purified synthetase preparation to catalyze formylation. A portion of the product was incubated with 10 mM CuSO$_4$ in 0.2 M sodium acetate, pH 5.5, for 20 min at 37°C. The tRNA was then precipitated with cold trichloroacetic acid and counted as described for the aminoacylation assay. The extent of formylation was determined by comparing the extent of aminoacylation before and after incubation with Cu$^{++}$.

**Analysis of nucleoside composition**

The nucleoside composition of tRNA and oligonucleotide preparations was analyzed by the tritium derivative method of Randerath et al. (1980).
RNA samples were first hydrolyzed to nucleosides by incubation for 6 h at 37°C in a mixture of 0.20 µg/µL ribonuclease A, 0.20 µg/µL snake venom phosphodiesterase and 0.16 µg/µL bacterial alkaline phosphatase in 30 mM sodium bicine buffer, pH 8.0, containing 10 mM MgCl₂. Following digestion, a portion of the mixture was oxidized with 1.2 mM NaIO₄ for 2 h at room temperature in the dark. The resulting nucleoside dialdehydes were reduced to ³H-labeled nucleoside trialcohols by incubation with 10 mM [³H]-KBH₄, 3Ci/mmol, for 2 h in the dark at room temperature. Excess borohydride was then destroyed by addition of 5 N acetic acid, and the samples were dried over KOH pellets in a vacuum desicator.

The mixture of ³H-labeled nucleoside trialcohols was separated by two dimensional chromatography on a cellulose thin-layer sheet. Development of the first dimension was with a solvent system composed of acetonitrile, ethyl acetate, 1-butanol, 2-propanol, and 6 N ammonium hydroxide (7:2:1:1:2.7). After drying, a wick of Whatman #1 paper was attached to the top of the second dimension and the plates were then developed with a solvent system composed of t-amyl alcohol, methyl ethyl ketone, acetonitrile, ethyl acetate, H₂O, and formic acid (40:20:14:20:14:1.8).

The separated tritium-labeled nucleoside derivatives were located by low-temperature fluorography. Chromatograms were coated with 16 mL of 7% PPO in ethyl ether, and stapled to X-ray film. The films were exposed for 24-72 h at -80°C. Developed films were then used to locate the ³H-labeled nucleoside trialcohols whose identity was determined by their migration pattern (Randerath et al., 1980). The labeled nucleoside derivatives were excised and extracted from the chromatogram with 2M aqueous ammonia, and quantitated by liquid scintillation counting. Because this procedure results in a stoichiometric incorporation of tritium into the nucleosides, the base composition of the RNA can be determined from the radioactivity of the individual nucleoside trialcohols.
Polyacrylamide gel electrophoresis

Electrophoresis of tRNA samples was carried out in 15% polyacrylamide gels (acrylamide:bisacrylamide, 20:1) prepared in electrophoresis buffer consisting of 100 mM Tris-borate, pH 8.3, 2 mM EDTA containing 5M urea. Dimensions of the gels were either 1.5 x 120 x 150 mm (slab gels), or 0.4 x 300 x 310 mm (sequencing gels). Gels were polymerized by addition of 0.1% (w/v) ammonium persulfate and 0.01% N,N',N'-tetramethylethylenediamine. RNA samples were mixed with an equal volume of electrophoresis buffer containing 0.1% bromophenol blue and 0.1% xylene cyanol in 8M urea, heated to 90°C for 30 sec, and applied to the gel using glass capillary tubes. Electrophoresis was carried out at room temperature in the same buffer without urea for 2-6 h at 12 mA for small gels, or at 1000-1500 V for sequencing gels. RNA bands in the gels were detected by autoradiography using X-ray film, for radioactively labeled tRNAs, or by staining overnight with 1% pyronin Y in 15% (v/v) acetic acid followed by destaining in several changes of 7.5% (v/v) acetic acid, for unlabeled samples.

5' End labeling tRNA with $^{32}$P

Transfer RNA was labeled at the 5' end using polynucleotide kinase and [$\gamma$-$^{32}$P]ATP. The RNA was first dephosphorylated, by treatment with calf intestinal phosphatase, in 10 μL reactions containing 160 pmol of RNA, 50 mM Tris-HCl, pH 8.0, and 0.1 units of phosphatase. Reactions were incubated 30 min at 55°C, then terminated by addition of 5 mM nitrilotriacetic acid followed by incubation for 20 min at room temperature and then heating to 100°C for 3 min. For 5'-end labeling, 80 pmol of dephosphorylated RNA was dissolved in 10 μL containing 25 mM Tris-HCl, pH 8.0, containing 12.5 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM spermidine, 100 pmol [$\gamma$-$^{32}$P]ATP (2500 Ci/mmol), and 1 unit of T4 polynucleotide kinase. The reactions were incubated for 30 min at 37°C, and terminated by heating to 100°C for 3 min.
5'-$^{32}$P-tRNA was purified by preparative polyacrylamide gel electrophoresis in 15% polyacrylamide/8M urea gels (1.5 x 120 x 150 mm) as described in the previous section. Twenty to 40 pmol of labeled RNA were loaded into each lane. A strip of DEAE-cellulose paper was placed beneath the slab gel in the anode buffer chamber to trap unincorporated [$\gamma-^{32}$P]ATP as it ran off the bottom of the gel. Following electrophoresis, radioactive ink was applied to the corners of the gel and autoradiography was used to locate intact labeled tRNA. The gel was covered with plastic wrap and exposed to X-ray film for 30 sec. The developed X-ray film was then used as a template to cut out the band of intact tRNA from the gel.

$^{32}$P-Labeled tRNA was recovered for the polyacrylamide gel by a crush and soak procedure. The excised gel slice was placed in a 1.5 mL plastic centrifuge tube and crushed into a paste using a siliconized glass rod. One-half mL of elution buffer, consisting of 500 mM ammonium acetate, 0.1% sodium dodecyl sulfate, 0.001% Triton X-100, and 40 μg/mL unlabeled carrier tRNA, was added and the mixture was incubated overnight at room temperature with agitation. Polyacrylamide was removed by filtration through a quick-sep column (Isolab QS-P) and the extracted RNA was precipitated by addition of 1.0 mL cold ethanol. After incubation at $-70^\circ$C for 1 h, the precipitated RNA was recovered by centrifugation for 10 min at 14,000 x g in a microcentrifuge, washed with 0.5 mL of 95% ethanol, dried, and resuspended in 20 μL of water.

**Aniline cleavage of tRNA**

Aniline buffered at pH 4.5 with acetic acid induces strand scission at depurinated or depyrimidinated sites in RNA by catalyzing a $\beta$-elimination reaction at the position of the damaged base (Peattie, 1979). Localization of the open-ring, modified fluorodihydrouracil residue in the A form of FUra-substituted tRNA was accomplished using aniline treatment.
and analysis of the resulting fragments by electrophoresis on sequencing gels. Samples containing ca. 1 μCi of 5'-32P end-labeled tRNA and 2 μg of unlabeled carrier tRNA were incubated in 20 μL of 1 M aniline/acetate buffer, pH 4.5, at 60°C for 30 min. Reactions were terminated by freezing at -70°C, and lyophilizing to dryness. The samples were then redissolved in 20 μL of water, frozen and lyophilized to dryness twice more to remove all traces of aniline/acetate. The reactions were analyzed by polyacrylamide gel electrophoresis on sequencing gels as described previously. Control samples were incubated in 50 mM sodium acetate, pH 4.5, without aniline.

Ribonuclease H reactions

Conditions for RNase H digestions were based primarily upon those of Donis-Keller (1979) as modified by T. G. Lawson and H. L. Wieth of Purdue Univ. (personal communication). Each reaction was 10 μL in volume and contained (unless otherwise indicated) ca. 0.3 pmol of 5'-32P end-labeled tRNA, 30-120 pmol of synthetic dGTAA, 40 mM Tris-HCl, pH 7.9, 4 mM MgCl₂, 1 mM dithiothreitol, 30 μg/mL bovine serum albumin, and 0.5 units of RNase H. Control reactions included all components except the deoxyoligonucleotide or RNase H. Digestions were allowed to proceed for 30 min at 30°C and terminated by freezing at -70°C, after addition of 10 μL of 8M urea containing 0.1% bromophenol blue and 0.1% xylene cyanol. Samples from the RNase digestion were analyzed by electrophoresis on polyacrylamide gels as described previously.

Limited ribonuclease T1 digestion of tRNA

A partial RNase T1 digestion of 5'-32P-tRNA was used to generate a nested set of labeled fragments, each extending from the 5'-end to an internal guanosine residue. The procedure was based on that of Donis-Keller et al. (1977). Approximately 1 μCi of labeled tRNA was incubated
in 5 μL of a mixture consisting of 20 mM sodium citrate, pH 5.0, 1 mM EDTA, 7 M urea, 0.45 μg/μL unlabeled carrier tRNA, 0.025% bromophenol blue, 0.025% xylene cyanol, and either 0.4 or 0.04 units of RNase T1. Control reactions included all components except the ribonuclease. The mixtures were incubated at 50°C for 15 min and the digestion stopped by immersion in ice.

**Bisulfite modification of FUra-substituted tRNA**

The conditions for bisulfite modification of (FUra)Val were those described by Schulman and Pelka (1977). FUra-substituted tRNAVal was dissolved in 2M sodium bisulfite, pH 7.0, containing 10 mM MgCl2, to a final concentration of 30 A260/mL. Controls samples were dissolved in 10 mM Tris-HCl, pH 7.0, with 10 mM MgCl2. The samples were incubated at room temperature (27°C) for 4 h. After addition of two volumes of H2O, the tRNA solutions were dialyzed twice against 500 mL of 0.15M NaCl in 20 mM sodium acetate, pH 4.5, for a total of 16 h. The RNA was then precipitated by addition of two volumes of ethanol and prepared for 19F NMR in the usual manner.

**Photoreaction of psoralen with FUra-containing tRNA**

Thirty mL samples containing equimolar concentrations of 4'-hydroxy-methyl-4,5',8-trimethyl psoralen (HMT) (20 μg/mL) and FUra-substituted tRNA (200 μg/mL) in 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA were placed in Pyrex glass tubes. Samples were deoxygenated by flushing with nitrogen for 1 h, and irradiated at 365 nm for 5 min in a Rayonet photoreactor equipped with RUL 3500-A lamps. The temperature of the sample was ca. 5°C After irradiation, the samples were extracted twice with two volumes of chloroform/isoamyl alcohol (24:1, v/v), three times with ether, and then ethanol precipitated three times to remove unbound HMT.
Oligonucleotide synthesis

\[ G_pU + nADP \rightleftharpoons G_pU(pA)_n + nPi \]

is reversible, resulting in phosphorolysis of the ribooligonucleotides. The chain length distribution of oligonucleotides synthesized is, therefore, determined by the ratio of nucleotide-5'-diphosphate to primer originally present in the reaction mixture, provided that the mixture is incubated long enough to reach equilibrium. The conditions used to obtain optimal synthesis of tri- and tetranucleotides were 7.5 mM primer, 20 mM ADP, 10 mM MgCl₂, 0.4 M NaCl, 0.2 M Tris-HCl pH 9.3 and 1.2 mg/ml polynucleotide phosphorylase. After the reaction mixture had been incubated for 90 h at 34°C, the sample was heated to 90°C for 15 min to inactivate the enzyme. The volume of the mixture was increased two fold by addition of H₂O, and bacterial alkaline phosphatase (18 µg/mL) was added to hydrolyze any unreacted ADP. \( C_pG_pA_pA \) was synthesized in the same manner using \( C_pG \) as primer.

The resulting oligonucleotides were separated on a DEAE-cellulose column (1.5 x 15 cm) using a 2 x 400 mL linear gradient containing 5-500 mM triethylammonium bicarbonate (TEAB) pH 8.0. An example of the elution profile obtained for \( G_pU(pA)_n \) synthesis is shown in Figure 4. Each successive peak represents an oligonucleotide containing one more adenylate residue than the preceding one. The tri- and tetranucleotides were further purified on a DEAE-Sephadex A-25 column (0.9 x 20 cm), using a 2 x 100 mL linear gradient containing either 0.05-0.5 M or 0.1-1.0 M TEAB. Fractions containing the desired products were evaporated to dryness, and triethylammonium bicarbonate was removed by repeated addition of 50% methanol and evaporation. In the final step, the oligonucleotides
Figure 4. DEAE-cellulose ion exchange chromatographic separation of products from the polynucleotide phosphorylase reaction of G\textsubscript{p}U with ADP. The column was eluted at room temperature with a linear gradient of 5 to 500 mM triethylammonium bicarbonate (TEAB), pH 8.0, at a flow rate of 1.0 mL/min.
were converted to the ammonium salt by passage over Dowex 50W-X8(NH₄⁺) and then lyophilized to dryness. Yields were 26% for G₃U₃₅₋, and 12% for both G₃U₃₅₋₃ and C₃G₃₅₋₃, based on the dinucleoside monophosphate primer.

Ap₃₅₋₃ was prepared by a ribonuclease T₃ assisted, polynucleotide phosphorylase catalyzed addition of GDP to Ap₃₅₋₃ (Mohr and Thatch, 1969). Reaction conditions were 7 mM primer, 35 mM GDP, 0.2 M Tris-HCl, pH 8.3, 10 mM MgCl₂, 0.5 mg/mL polynucleotide phosphorylase, and 300 units/mL of ribonuclease T₃. The reaction was incubated 24 h at 34°C and the products were purified as described above. The yield of Ap₃₅₋₃ was 76%.

The tetranucleotide G₃U₃₅₋₃G was prepared by elongating G₃U₃₅₋₃ with GDP using polynucleotide phosphorylase, as described by Uhlenbeck et al. (1970). Reaction conditions were those described for G₃U₃₅₋₃ synthesis except that the concentration of G₃U₃₅₋₃ as primer was 1.0 mM, GDP was 0.5 mM, and the enzyme concentration was 0.3 mg/mL. The reaction was incubated for 2 h at 34°C before being heat inactivated and dephosphorylated as described above. Twenty-four percent of the GDP was incorporated into the tetramer.

The syntheses of G₃U₃₅₋₃₃ and G₃U₃₅₋₃₃U were accomplished by using T₄ RNA ligase to join G₃U₃₅₋₃ to the 3',5'-nucleoside bisphosphates p₃₅₋₃₃ and p₃₅₋₃₃U, respectively (England and Uhlenbeck, 1978). RNA ligase reactions were carried out in 1.0 mL containing 0.17-0.30 mM nucleoside bisphosphate as donor, 0.7-1.2 mM G₃U₃₅₋₃ as acceptor, 0.5 mM ATP, 20 mM MgCl₂, 50 mM HEPES, pH 8.3, 10 μg/mL bovine serum albumin, 3.3 mM dithiothreitol, and 50-150 units/mL T₄ RNA ligase. Reactions were incubated at 37°C for 4 h and then heated to 90°C for 2 min to inactivate the ligase. After diluting the sample two-fold with water, 150 μg/mL of bacterial alkaline phosphatase was added to hydrolyze the phosphate from the 3' end of the tetranucleotides. The tetranucleotide products were separated from unreacted trimer by chromatography on DEAE-cellulose as described for the purifica-
tion of G₃U₅A, except that the TEAB gradient was 50-400 mM. Yields were 31% for G₃U₅C and 21% for G₃U₅U.

The nucleoside composition of all the synthetic oligonucleotides used in these studies was confirmed by the tritium derivative method of Randerath et al. (1980) (scaled down version 2). The results of these analyses are shown in Table 1. In each case the composition of the purified oligonucleotide was within experimental error of that expected.

Table 1. Analysis of Synthetic Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Guanosine</th>
<th>Uracil</th>
<th>Adenosine</th>
<th>Cytosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>GpUpA</td>
<td>1.00</td>
<td>1.08±0.09</td>
<td>0.97±0.12</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td>GpUpApA</td>
<td>1.00</td>
<td>0.95±0.08</td>
<td>1.88±0.08</td>
<td>0.00±0.02</td>
</tr>
<tr>
<td>GpUpApC</td>
<td>1.00</td>
<td>1.28±0.02</td>
<td>1.04±0.01</td>
<td>1.16±0.06</td>
</tr>
<tr>
<td>GpUpApG</td>
<td>1.89±0.08</td>
<td>1.00</td>
<td>1.04±0.03</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>GpUpApU</td>
<td>1.00</td>
<td>2.22±0.016</td>
<td>1.10±0.08</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>CpGpApA</td>
<td>1.00</td>
<td>0.07±0.01</td>
<td>2.04±0.12</td>
<td>0.90±0.04</td>
</tr>
</tbody>
</table>

^Results are normalized to the value shown as 1.00.
^Standard deviation in duplicate analyses.

NMR spectroscopy

For ¹⁹F NMR spectroscopy, transfer RNA samples (2.5-5.5 mg) were dissolved in a minimum volume of the buffer to be used in each experiment, and were then dialyzed against two changes of at least 1000 volumes of the same buffer, using a flow dialysis microcell (BRL model #1200 MA). After dialysis, the volume of the sample was adjusted to 0.3 mL, and 10% (v/v)
$^2\text{H}_2\text{O}$ was added to serve as an internal lock signal. Standard conditions are 50 mM sodium cacodylate, pH 6.0, 15 mM MgCl$_2$, 100 mM NaCl, and 1 mM EDTA. The sample was then transferred to a Wilmad 529A-10 NMR microtube, which was suspended in a 10 mm NMR sample tube and surrounded with sample buffer containing 1 mM 5-fluorouracil as an external standard.

For studies at low divalent cation concentration, Mg$^{++}$ was removed from the tRNA by dissolving it in 20 mL of 10 mM EDTA, pH 7.0, heating to 70°C for 5 min, and allowing the sample to cool slowly to room temperature. The tRNA was then precipitated with ethanol, collected, dissolved in sample buffer that had been treated with Chelex 100, and then dialyzed against this buffer, as described above. Transfer RNA treated in this manner contained less than 0.1 mol of Mg$^{++}$ per mol of tRNA, as determined by atomic absorption measurements on a Perkin-Elmer 2000 AA spectrometer (kindly provided by Dr. E. Travis Littledike, National Animal Disease Center, Ames Iowa).

Magnesium, manganese, sodium, or spermine was added as small aliquots of a concentrated stock solution prepared in the same buffer as the tRNA solution. Oligonucleotides were added as lyophilized solids.

$^{19}$F NMR spectra were obtained at room temperature (unless otherwise noted) on a Bruker WM300 pulsed FT NMR spectrometer at 282 MHz. Spectra were collected by using 8K data points, with no relaxation delay and a 37° pulse to optimize the Ernst condition (Shaw, 1976), except when making area measurements, in which case a 5 sec relaxation delay and 90° pulse angle were used to ensure complete relaxation. Typically 10,000 to 30,000 scans were collected and 10 Hz spectral line broadening was used. Chemical shifts are reported relative to free 5-fluorouracil; shifts to higher shielding are indicated by negative numbers. Integration of peak areas was accomplished using DISNMR software with an Aspect 2000 microcomputer. After each set of experiments, the activity of the tRNA was checked by
aminoacylation assay, and degradation was monitored by polyacrylamide gel electrophoresis.

For $^1$H NMR experiments, transfer RNA was dissolved in 0.3 mL of 30 mM sodium phosphate, pH 6.0, 20 mM MgCl$_2$, 100 mM NaCl. The samples were then dialyzed against this same buffer in the manner described for $^{19}$F NMR. Transfer RNA was transferred to $^2$H$_2$O by twice lyophilizing the sample to dryness and redissolving it in 99.996% $^2$H$_2$O (Strohler Isotope Chemical). Oligonucleotides used for $^1$H NMR were prepared as described previously, then dissolved in $^2$H$_2$O and lyophilized to dryness prior to use. $^1$H NMR spectra were collected on a Bruker WM300 FT NMR as described previously for $^{19}$F NMR except that 16K data points were used. Proton chemical shifts are reported relative to DSS.
RESULTS

Purification and Partial Characterization of FUra-substituted tRNA$^{\text{Met}}_m$

The results to be presented involve studies with three purified FUra-substituted *E. coli* transfer RNAs: tRNA$^{\text{Val}}_l$, tRNA$^{\text{Met}}_f$, and tRNA$^{\text{Met}}_m$. Previous publications describe the preparation and properties of the valine tRNA (Horowitz et al., 1974) and initiator methionine tRNA (Hills et al., 1983). Elongator methionine tRNA, tRNA$^{\text{Met}}_m$, from FUra-treated cells was purified for the first time in the course of this research, and its preparation and some of its properties will be described.

**Purification of (FUra)tRNA$^{\text{Met}}_m$**

A summary of the purification of FUra-substituted tRNA$^{\text{Met}}_m$, as described under methods, is shown in Table 2. During chromatography on BD-cellulose, (FUra)tRNA$^{\text{Met}}_m$ is resolved into two components (Fig. 5A), designated form A and B according to their order of elution. This behavior is similar to that of (FUra)tRNA$^{\text{Met}}_f$, which is also resolved into two isoaccepting species on BD-cellulose (Hills et al., 1983). Purified (FUra)tRNA$^{\text{Met}}_m$ form A had a specific activity of 1275 pmol/A$^{260}$ and constituted 26% of the tRNA$^{\text{Met}}_m$ recovered (Table 2), while form B, had a specific activity of 1500 pmol/A$^{260}$ and made up 56% of the total (Table 2). (The remaining 18% of the (FUra)tRNA$^{\text{Met}}_m$ was an unresolved mixture of the two forms). Both isoacceptors activity is comparable to that of normal tRNA$^{\text{Met}}_m$, indicating that despite the massive substitution by 5-fluorouracil, this tRNA is fully capable of being aminoacylated. Neither isoacceptor could be formylated (less than 1%), and both forms migrated as a single band on 15% polyacrylamide gels (results not shown).

Reverse phase chromatography of (FUra)tRNA$^{\text{Met}}_m$ on RPC-5 gave only slight additional purification (Table 2). However, a partial resolution
of isoacceptor A into two peaks was noted (Fig. 5B). Isoacceptor B chromato­
graphed as a single broad peak on RPC-5 (Fig. 5C) with some indication
of heterogeneity. In both cases, the absorbance at 260 nm paralleled the
methionine acceptance activity. Similar behavior was also noted for chro­
matography of \((\text{FUra})\text{tRNA}^\text{Met}_\text{m}\) on RPC-5; two peaks were obtained with form A,
whereas form B chromatographed as a single peak (Hills et al., 1983).
These results suggest the existence of additional forms of both FUra-
substituted methionine tRNAs, and other evidence indicates additional
forms of FUra-substituted tRNA^Val\(^r\) (Hardin, 1984). The nature of these
additional forms, however, has not been investigated.

**Nucleoside analysis of \((\text{FUra})\text{tRNA}^\text{Met}_\text{m}\)**

The nucleoside composition of both forms of FUra-substituted \(\text{tRNA}^\text{Met}_\text{m}\),
as determined by the tritium derivative method (Randerath et al., 1980),
is shown in Table 3. As described previously for FUra-substituted valine
tRNA (Horowitz et al., 1974) and initiator methionine tRNA (Hills et
Figure 5. Chromatography of FUra-substituted tRNA\textsubscript{Met}.

(A) Separation of two forms (A and B) of (FUra)tRNA\textsubscript{Met} on BD-cellulose. Transfer RNA recovered from DEAE-Sephadex chromatography (see Methods) was applied to a BD-cellulose column. The column was developed with a linear NaCl gradient from 0.4 to 1.0 M. Fractions of 2.0 mL were collected at 24 mL/h.

(B) Chromatography of (FUra)tRNA\textsubscript{Met} form A on RPC-5.
Isoacceptor A from BD-cellulose was applied to an RPC-5 column and the column was then developed with a 0.5-1.0 M NaCl linear gradient. One mL fractions were collected at a flow rate of 1.4 mL/min.

(C) RPC-5 chromatography of (FUra)tRNA\textsubscript{Met} form B.
Isoacceptor B from BD-cellulose chromatography was chromatographed as described above for the A form. (o) Absorbance at 260 nm; (●) methionine acceptance activity; (▲) NaCl concentration
Table 3. Nucleoside composition of 5-fluorouracil-substituted tRNA$^{Met}_{m}$

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Expected # of residues</th>
<th>(A) Form</th>
<th>(B) Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td>18</td>
<td>17.5 ± 0.23$^a$</td>
<td>17.8 ± 0.22</td>
</tr>
<tr>
<td>Cystosine</td>
<td>19</td>
<td>20.3 ± 0.03</td>
<td>20.1 ± 0.03</td>
</tr>
<tr>
<td>Adenosine</td>
<td>18</td>
<td>17.8 ± 0.06</td>
<td>18.1 ± 0.20</td>
</tr>
<tr>
<td>Uracil</td>
<td>9+1$^b$</td>
<td>0.48 ± 0.03</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>18(17)$^c$</td>
<td>15.8 ± 0.09</td>
<td>16.8 ± 0.05</td>
</tr>
<tr>
<td>5-Fluorocytosine</td>
<td>0</td>
<td>0.61 ± 0.03</td>
<td>0.76 ± 0</td>
</tr>
<tr>
<td>Ribothymidine</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudouracil</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dihydouracil</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>m$^7$Guanosined$^d$</td>
<td>1</td>
<td>0.98 ± 0.05</td>
<td>0.92 ± 0.08</td>
</tr>
<tr>
<td>t$^6$Adenosine</td>
<td>1</td>
<td>0.13 ± 0.01</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>2'OmGuanosine</td>
<td>1</td>
<td>_e</td>
<td>_e</td>
</tr>
<tr>
<td>ac$^4$Cytosine</td>
<td>1</td>
<td>_f</td>
<td>_f</td>
</tr>
<tr>
<td>x$^e$</td>
<td>1</td>
<td>_f</td>
<td>_f</td>
</tr>
<tr>
<td>s$^4$Uracil$^g$</td>
<td>1</td>
<td>_g</td>
<td>_g</td>
</tr>
</tbody>
</table>

$^a$Standard deviation in duplicate analyses.

$^b$s$^4$Uracil is converted to uracil during labeling procedure.

$^c$18 residues (B) form, 17 residues (A) form (see text).

$^d$Corrected for 64% recovery.

$^e$Can't be determined by the $^3$H-labeling method (Randerath et al., 1980).

$^f$Not determined.

$^g$X is 3N-(3-amino-3-carboxypropyl) uracil.
nearly all the uracil and uracil-derived modified nucleosides of \((\text{FURA})t\text{RNA}^\text{Met}_m\) are replaced by 5-fluorouracil (Table 3). More than 95% of the uridine is replaced by FURA, and the residual levels of dihydrouridine, pseudouridine, and ribothymidine are virtually undetectable. Values obtained for the other major nucleosides (adenosine, guanosine, and cytosine) are all very close to the number of residues expected (Table 3 and Figure 2), although the value for cytosine is slightly high. Approximately 3% of the cytosine in \((\text{FURA})t\text{RNA}^\text{Met}_m\) is replaced by 5-fluorocytidine. Similar levels of 5-fluorocytidine were also noted in FURA-substituted tRNA$_1^{\text{Val}}$ (Horowitz et al., 1974) and \((\text{FURA})t\text{RNA}^\text{Met}_f\) (Hills et al., 1983). The nonuridine-derived modified nucleoside t$_A^6$ was present at less than 20% of the normal level.

The only significant difference in the nucleoside composition of the two isoacceptors of \((\text{FURA})t\text{RNA}^\text{Met}_m\) is that form B contains one more FURA than form A. Previously, it was shown that the two isoaccepting forms of FURA-substituted initiator methionine tRNA differ only in that form A contains a modified 5-fluoro-5,6-dihydrouridine at position 20 (normally occupied by dihydrouridine), whereas form B contains 5-fluorouridine at this position (Hills et al., 1983). Fluorodihydrouridine is extremely alkali-labile and undergoes a ring-opening reaction to yield fluoroureido-propionic acid during chromatography on DEAE-cellulose (at pH 8.9) [used to separate normal from FURA-substituted tRNA] (Horowitz et al., 1983). Therefore, the A form of \((\text{FURA})t\text{RNA}^\text{Met}_f\) contains one less FURd residue than form B (Hills et al., 1983). The results in Table 3 suggest that a similar situation exists with FURA-substituted tRNA$_m^{\text{Met}}$.

19F NMR spectra of FURA-substituted tRNA$_m^{\text{Met}}$

19F NMR spectra of both isoacceptors of 5-fluorouracil-substituted tRNA$_m^{\text{Met}}$, recorded at pH 6.0 in 15 mM MgCl$_2$ and 100 mM NaCl at room temper-
Figure 6. $^{19}$F NMR spectra of 5-fluorouracil-substituted tRNA$^{\text{Met}}_{m}$. (A) Isoacceptor A (0.50 mM). (B) Isoacceptor B (0.60 mM). The tRNA samples were dissolved in standard buffer (see Methods) and the spectra were recorded at 25°C. Areas of each region of the spectrum were obtained by integration on the spectrometer and were normalized to the area of peak D. Peak designations are also indicated.
ature, are shown in Figure 6. Thirteen resolved resonances and two shoulders are visible in the spectrum of form B, corresponding to the 18 FUra residues in the analogue substituted tRNA (see cloverleaf insert in Fig. 6). Relative areas of each region of the spectrum, obtained by integration on the spectrometer and normalized to the area of peak D, are also indicated. Due to the large number of peaks and the degree to which many of them overlap, the exact location of all the $^{19}$F signals in this spectrum is difficult to determine, and some questions remain regarding the exact number of resonances in the region between 5 and 7 ppm, and between 1.5 and 2.5 ppm.

The $^{19}$F spectrum of the A form of FUra-substituted tRNA$^{\text{Met}}_m$ differs from that of the B form mainly by the shift of one resonance from 4.6 ppm (peak F) in isoacceptor B to -15 ppm in isoacceptor A (Fig. 6). As described previously, similar differences exist between the $^{19}$F NMR spectra of the A and B forms of FUra-substituted tRNA$^{\text{Met}}_m$ and of (Fig. 3B) (FUra)$tRNA^{\text{Val}}_1$ (3A). The $^{19}$F signal at -15 ppm has been shown to be due to a ring-opened fluorodihydrouridine substituent in the A form of FUra-substituted tRNA (Horowitz et al., 1983). The difference between the $^{19}$F spectra of the two isoacceptors of (FUra)$tRNA^{\text{Met}}_m$ is also likely due to the presence of a modified fluorodihydrouridine. The position of this modified FUra will be discussed in the next section.

A comparison of the $^{19}$F NMR spectrum of (FUra)$tRNA^{\text{Met}}_m$ (Fig. 6) with the previously obtained spectra of FUra-substituted tRNA$^{\text{Val}}_1$ (Fig. 3A) and tRNA$^{\text{Met}}_f$ (Fig. 3B) shows that there are similarities among all three, as well as some unique features in the tRNA$^{\text{Met}}_m$ spectrum. Although the range of chemical shifts for the $^{19}$F resonances is similar in all three spectra (ca. 0.5 to 8.0 ppm downfield from FUra), the $^{19}$F spectrum of (FUra)$tRNA^{\text{Met}}_m$ contains a resonance farther upfield, at 0.3 ppm, than any resonance in the other two spectra. The spectrum of (FUra)$tRNA^{\text{Met}}_m$ also
contains at least four resonances in the downfield portion of the spectrum (5.5-8.0 ppm) whereas spectra of fluorinated tRNA<sub>Val</sub> and tRNA<sub>Met</sub> show only three peaks in this area.

**Determination of the position of fluorodihydrouridine in (FUra)tRNA**

The modified fluorodihydrouridine in (FUra)tRNA<sub>Met</sub> has been located at position 20 in isoacceptor A by sequence analysis of both forms of this analogue-substituted tRNA (Hills et al., 1983). However, the location of the modified FUra in (FUra)tRNA<sub>Val</sub> and (FUra)tRNA<sub>Met</sub> has not been determined. To determine the position of the modified FUra in the A form of these tRNAs, both isoacceptors of each tRNA, labeled at their 5' ends with <sup>32</sup>P (see Methods), were treated with aniline buffered at pH 4.5. Modification of 5-fluorouracil to fluorodihydrouracil and the subsequent alkali-catalyzed ring-opening of the modified base (during chromatography on DEAE-cellulose at pH 8.9) renders the tRNA susceptible to aniline induced strand scission at the position of this residue. The cleavage products were then analyzed by polyacrylamide gel electrophoresis. Both forms of (FUra)tRNA<sub>Val</sub> were also analyzed by this method.

Positions of aniline cleavage were determined by comparing the cleavage sites to a partial ribonuclease T1 digest ladder. The mobility of bands corresponding to RNase T1 fragments do not coincide exactly with those produced by aniline cleavage because oligonucleotides produced by RNase T1 terminate in a 3'-phosphate, whereas fragments generated by aniline cleavage terminate in an aniline-substituted 3'-ribose (Maxam, 1983). As a result, the aniline cleavage fragments are retarded by approximately one nucleotide interval relative to the corresponding RNase T1 fragments.

As shown in Figure 7, the major cleavage site observed in the A form of each tRNA that is not present indicates the position of the fluorodi-
Figure 7. Localization of 5-fluorodihydrouridine in FUra-substituted tRNAs. Autoradiograms of 15% sequencing gel analysis of fragments derived by aniline (pH 4.5) cleavage of the A and B forms of 5'-32P-labeled 5-fluorouracil-substituted tRNA^Met^, tRNA^Val^, and tRNA^Met^. Control samples were incubated in 50 mM sodium acetate, pH 4.5. All incubations were at 50°C for 20 min. A ribonuclease T1 sequencing ladder (see Methods) was used to identify the sites of cleavage.
hydrouridine residue. In (FUra)\(tRNA^{\text{Met}}_m\), the site of the modified FUra is located at position 20, confirming previous findings (Hills et al., 1983). The fluordihydrouridine is located at position 17 in (FUra)\(tRNA^{\text{Val}}_m\) and at position 20 in (FUra)\(tRNA^{\text{Met}}_m\) (Fig. 7). [A second minor band, with mobility slightly faster than the major band, is also observed in the A form of each tRNA, possibly due to a partial loss of the aniline moiety from the 3' end of the fragment (Maxam, 1983)]. Aniline also cleaved both forms of the methionine tRNAs at \(m^7G-47\).

The modified fluorodihydrouridine in the A form of each tRNA is located in the D loop (Fig. 2) at a position normally occupied by 5,6-dihydrouridine. The D loop of \(tRNA^{\text{Met}}_m\) normally contains 3-4 dihydrouracils (Cory and Marcker, 1970). All of these sites are replaced by fluoropyrimidines (Table 3), but only the FUra at position 20 in the A-form is reduced to fluorodihydrouracil.

19F NMR Studies of the Solution Structure of FUra-substituted tRNA

To understand the function of tRNA, knowledge of the dynamic structure of the molecule in solution is essential. Using 19F NMR effectively as a probe of tRNA conformation requires an understanding of the environmental factors which influence the 19F spectra of FUra-substituted tRNAs. These studies were undertaken to characterize the effect of pH, ionic environment, temperature, and chemical modification on the spectra. Specific assignment of the 19F signals in the spectra to individual FUra residues in the tRNA molecule is necessary before spectral changes can be interpreted at the molecular level, and these experiments represent an initial approach to the assignment problem.
Effect of pH on the $^{19}$F NMR spectrum of FUra-substituted tRNA

Ionization produces large downfield shifts of the $^{19}$F NMR resonance of 5-fluorouracil. Dissociation of the N(3) proton of 5-fluorouridine shifts the $^{19}$F signal over 1.6 ppm downfield (Hardin, 1984), and Alderfer et al., (1983) have reported a downfield shift of nearly 2.7 ppm as a result of the ionization of poly(FU).

Figure 8A shows the effect of changing pH on the $^{19}$F NMR spectrum of FUra-substituted tRNA$^{Val}$, and the pH dependence of the chemical shift of each peak is shown in Figure 8B. Changing the pH from 4.5 to 8.75 produces differential effects on the chemical shifts of the various peaks in the spectrum. The most pronounced effect of increasing pH is on $^{19}$F resonances in the central portion of the spectrum (3.8 to 5.5 ppm). These peaks shift downfield 1.0-2.5 ppm over the pH range of 6.0 to 8.75. This behavior is similar to that of 5-fluorouridine (Hardin, 1984) and indicates that these residues have pKas close to that of free FUrd. One resonance in this region, labeled peak G, is unaffected by changing pH from 4.5 to 8.75.

The $^{19}$F resonances in the upfield portion of the spectrum (above 3.8 ppm) are largely unperturbed by changes in pH, indicating that the N(3) proton of these FUra residues has not dissociated even at pH 8.75. The farthest downfield resonances (peaks A and B) also do not change chemical shift significantly between pH 4.0 and 8.75.

Some interesting changes in the spectrum occur at low pH. As the pH is raised from 4.5 to 6.0, peak C shifts downfield and peak F moves upfield. These effects may reflect conformational changes in the tRNA rather than dissociation of the N(3) proton, especially in the case of peak F, which shifts upfield with increasing pH, because this movement is the opposite of that seen with dissociation of N(3)-H. Because 5-fluoro-uridine has a pKa of 7.57 (Wempen et al., 1961), $^{19}$F NMR spectra of FUra-
Figure 8. Effect of pH on (FUra)tRNA$_{Val}$.

(A) $^{19}$F NMR spectra of (FUra)tRNA$_{Val}$ at various pHs. The tRNA (0.22-0.40 mM) was dialyzed against buffers of the indicated pH values. Buffers used were 50 mM sodium cacodylate containing 100 mM NaCl and 15 mM MgCl$_2$ (pH 4.5-7.50) and 5 mM Tris-HCl containing 150 mM NaCl and 15 mM MgCl$_2$ (pH 8.0-8.75). Both buffers also contained 1 mM EDTA, and 10% $^2$H$_2$O as an internal lock.

(B) The pH dependence of the chemical shifts of $^{19}$F resonances identified in (A)
CHEMICAL SHIFT (PPM FROM FUra)
Figure 8. (Continued)
substituted tRNA were usually recorded at pH 6.0 to minimize effects due to ionization of the incorporated FUra.

**19F NMR studies of ion binding to FUra-substituted tRNA**

The conformation of transfer RNA is sensitive to the concentration of cations such as magnesium and sodium, which serve to stabilize the folded three-dimensional structure of this negatively charged polynucleotide (for review see Schimmel and Redfield, 1980). In addition, spermine and magnesium affect many of the biological activities of tRNA, including aminoacylation (Loftfield et al., 1981) and protein synthesis (Thompson et al., 1981). Changing the concentration of cations also affects the 19F NMR spectra of FUra-substituted tRNAs. Characterization of these effects may aid in making peak assignments in the spectra, and eventually these studies may yield valuable information regarding the conformational states of tRNA in solution.

**Magnesium ions**

As shown in Figure 9A, magnesium has a large effect on the 19F NMR spectrum of FUra-substituted tRNA\textsubscript{Val}. The tRNA sample used to obtain the spectrum labeled 0 Mg\textsuperscript{++} contained less than 0.1 Mg\textsuperscript{++}/tRNA as determined by atomic absorption spectroscopy. By titrating the tRNA solution with added Mg\textsuperscript{++} it is possible, in most cases, to identify which resonances in the absence of magnesium correspond to signals in the presence of Mg\textsuperscript{++}. These peaks are labeled in Figure 9A, and Figure 9B shows the affect of Mg\textsuperscript{++} on the chemical shift of the individual resonances in the spectrum. In some cases, ambiguities arise due to peaks overlapping. In these situations, it was generally assumed that trends in shift patterns are continuous. Also, in some instances the properties of certain 19F resonances, such as shifts induced by codon binding (see later Results), were used to aid in making peak identifications.

Most of the 19F peaks shift downfield as the concentration of magnesium is increased. The largest effect occurs with peak A, which moves
Figure 9. Effects of magnesium on (FUra)tRNA\textsuperscript{Val}.

(A) $^{19}$F NMR spectrum of (FUra)tRNA\textsuperscript{Val} at various levels of magnesium. Magnesium was removed from the tRNA (<0.1 Mg$^{++}$/tRNA) as described under Methods, and the tRNA sample (0.44 mM) was dissolved in standard buffer lacking Mg$^{++}$. MgCl\textsubscript{2} was then added to give the indicated molar ratios of Mg$^{++}$/tRNA.

(B) Magnesium ion dependence of the chemical shifts of $^{19}$F resonances identified in (A). D indicates peak positions when the sample is dialyzed against buffer containing 15 mM MgCl\textsubscript{2}. 
Figure 9. (Continued)
over 3 ppm downfield as the Mg$^{++}$ ratio is increased from 0 to 50/tRNA. This resonance is also the most sensitive to changes in the NaCl concentration (Fig. 10). Peaks J and K also exhibit large shifts (over 1 ppm) in this range of Mg$^{++}$. These shifts all start at the lowest magnesium level, but the changes reach completion at different levels of Mg$^{++}$. Peak A shifts in a continuous manner over the entire range of [Mg$^{++}$], and the process does not appear to be complete by 50 Mg$^{++}$/tRNA. The downfield movement of peak K is completed by 40 Mg$^{++}$/tRNA, whereas peak J stops shifting by 20 Mg$^{++}$/tRNA.

Two resonances in the central portion of the spectrum, peaks G and H, also shift 0.2 and 0.5 ppm downfield, respectively, as the Mg$^{++}$/tRNA ratio is raised from 0 to 20; no further shifts are observed for these peaks with increasing Mg$^{++}$. Peak F demonstrates an unusual behavior. This resonance remains at 3.8 ppm until the Mg$^{++}$ level is raised above 30/tRNA; it then shifts downfield to 4.3 ppm as the Mg$^{++}$ concentration is increased. The movements of peaks F, G, and H are difficult to follow in the central portion of the spectrum because all three overlap when the magnesium concentration is between 20 and 30/tRNA. Peaks F and H could, however, be identified on the basis of their behavior on binding of tri- and tetrancleotide codons to (FUra)tRNA$^{Val}$ (see Fig. 28). These resonances are also the most temperature sensitive peaks in the spectrum in the absence or presence of magnesium (see Figure 16; also Hardin, 1984).

Addition of 50 Mg$^{++}$/tRNA restores the $^{19}$F NMR spectrum of (FUra)tRNA$^{Val}$ to that observed when the sample is dialyzed against 15 mM MgCl$_2$ (compare Fig. 9A with 3B; also see Fig. 9B). The only exception is peak A which is found farther downfield in the dialyzed sample. This result indicates that the effects of Mg$^{++}$ removal are reversible.

The results obtained upon addition of Mg$^{++}$ to (FUra)tRNA$^{Val}$ (Fig. 9) were, in general, similar to those obtained previously by Hardin (1984).
However, improved resolution in the NMR spectra has allowed new interpretation of the current data.

**Sodium ions** The effect of sodium concentration, in the presence of 15 mM Mg**, on the $^{19}$F NMR spectra of FUra-substituted tRNA$_{Val}$, tRNA$_{Met}$, and tRNA$_{Met}^m$ is shown in Figure 10, and the dependence of the $^{19}$F chemical shifts for each resonance from all three tRNAs is plotted in Figure 11. In each case, the farthest downfield peak at low ionic strength (peak A) shifts upfield dramatically (over 2 ppm) as the NaCl concentration is raised from 0 to 500 mM. This behavior may indicate that the downfield resonance in each spectrum corresponds to a residue in a conserved structural feature of these three tRNAs. A second resonance, in the central portion of each spectrum, also shifts upfield approximately 0.8 ppm as the [NaCl] is increased. These peaks are labeled F, G, and I in the spectra of FUra-substituted tRNA$_{Val}$, tRNA$_{Met}$, and tRNA$_{Met}^m$, respectively, and may also be derived from FUra residues in a conserved feature of the three tRNAs (see Discussion).

In (FUra)tRNA$_{Val}$, two additional $^{19}$F resonances also shift slightly upfield with increasing ionic strength: peak K, originally at 2.8 ppm, and peak N, the farthest upfield resonance. The remaining $^{19}$F resonances in all three spectra are fairly insensitive to changes in the concentration of NaCl. The results obtained with (FUra)tRNA$_{Val}$ are similar to those described previously by Hardin (1984); however, improved resolution of the $^{19}$F NMR spectra has allowed new interpretation of the affects on peaks F and K.

Physical studies of transfer RNA have shown that at ionic strengths greater than 0.1M and moderate temperature (< 30°C), tRNA occurs in a "native" form even in the absence of magnesium (for review see Crothers and Cole, 1978). Gorenstein and Goldfield (1982) have shown that in 0.2 M Na$^+$ at 25°C, the $^{31}$P NMR spectra of several tRNAs are quite similar in the
Figure 10. Effects of NaCl on the $^{19}$F NMR spectra of FUra-substituted tRNA in the presence of magnesium (15 mM MgCl$_2$). tRNA$^{Val}_{1}$ (0.44 mM), tRNA$^{Met}_{f}$ (0.40 mM), or tRNA$^{Met}_{m}$ (0.34 mM) were dissolved in standard buffer (see Methods) containing the indicated NaCl concentrations.
Figure 11. Plot of the sodium chloride concentration dependence of the chemical shifts of $^{19}$F resonances identified in Figure 10
absence or presence of 10 mM Mg**, indicating that under these conditions the structure of the phosphodiester backbone of the tRNA remains largely unchanged even upon removal of Mg**. However, some differences were noted between the low and high Mg** spectra. These differences led the authors to suggest that at 19°C, 64% of the structure remains native while at 39°C, only 21% of the native structure is present in the absence of magnesium (Gorenstein and Goldfield, 1982).

The effects of Na* on the 19F NMR spectrum of (FUra)tRNA^Val at 24°C and in the absence of Mg** are shown in Figure 12. As the NaCl concentration is decreased, the downfield resonances, peaks B and C, are the most affected. Peak B splits into two components when the [NaCl] is below 150 mM and eventually splits into three broad peaks with diminished intensity when the ionic strength falls below 35 mM NaCl. Peak C shifts upfield into the central cluster of peaks as the salt concentration drops. The upfield group of signals (0.5-2.5 ppm) also appears to decrease in intensity while the intensity of the resonances in the central region of the spectrum increases as the ionic strength is reduced. These results resemble those observed on thermal denaturation of (FUra)tRNA^Val at 100 mM NaCl, in the absence of Mg** (Hardin, 1984), and likely represent unfolding of the tRNA molecule at low ionic strength.

Even at NaCl concentrations of up to 0.4M, the spectrum is not the same as that observed in the presence of 15 mM MgCl2 (compare Fig. 12 with 3A). This result indicates that Na* cannot completely replace Mg** in determining the solution structure of transfer RNA.

Manganese ions Manganese can substitute for magnesium, both in terms of the structure and functions of tRNA (Hyafil and Blanquet, 1977). The paramagnetic Mn** ion has been used in a number of NMR studies to probe divalent metal binding sites in tRNA molecules in solution (Chao and Kearns, 1977; Hurd et al., 1979; Gorenstein and Goldfield, 1982). The
Figure 12. Effects of NaCl on the $^{19}$F NMR spectrum of (FUra)tRNA$_{Val}$ in the absence of Mg$^{++}$. The tRNA (0.40 mM) was dissolved in 50 mM sodium cacodylate buffer, pH 6.0, containing the indicated NaCl concentrations (mM).
large magnetic moment of Mn**, due to the unpaired electron, affects the relaxation times of nearby nuclei, resulting in broadening of the NMR resonances from these nuclei. The extent of broadening is determined both by the distance between the bound manganese ion and the nucleus, and the residence time of the manganese at the binding site (Eisinger et al., 1965). Sites of Mn** binding in tRNA have been identified in the crystal structure of tRNA\textsuperscript{Phe} (Jack et al., 1977). Determination of which resonances in the \(^{19}\text{F}\) NMR spectrum are perturbed by Mn**, may aid in assigning these peaks to FUra bases near these binding sites.

The effect of manganese on the \(^{19}\text{F}\) NMR spectrum of FUra-substituted tRNA\textsubscript{Val} is presented in Figure 13. Selective paramagnetic line broadening effects are observed for peaks A, B, J, L, and N on addition of up to 0.04 Mn** per tRNA\textsubscript{Val} molecule, in the presence of 15 mM Mg**, 0.1 M NaCl, 50 mM sodium cacodylate, pH 6.0, and no EDTA. The largest effects occur with peaks J, L, and N. Broadening of these signals is detectable by 0.001 Mn**/tRNA\textsubscript{Val}, and is quite severe when the ratio of Mn** to tRNA reaches 0.04. Lesser effects are observed for peaks A and B, which begin to broaden when 0.007 Mn**/tRNA have been added. The remaining \(^{19}\text{F}\) peaks in the spectrum of (FUra)tRNA\textsubscript{Val} show little effect up to 0.04 Mn**/tRNA.

It is interesting that two of the \(^{19}\text{F}\) signals that are broadened by Mn**, peaks A and J, are also highly sensitive to the concentration of magnesium (see Fig. 9), whereas the remaining three Mn** sensitive peaks are among the least affected by Mg**. The level of Mn** required to broaden specific \(^{19}\text{F}\) resonances is very low. Broadening of some peaks can be detected when the tRNA to manganese ratio is 1000 to one, and the magnesium to manganese ratio is over 20,000 to one.

**Spermine** The interaction of spermine with (FUra)tRNA\textsubscript{Val} was also examined. This polyamine can replace magnesium, at least partially, in stimulating the biological activity of tRNA in vitro (Igavashi and Takeda,
Figure 13. Effects of manganese on the $^{19}$F NMR spectrum of (FUra)tRNA$_{Val}$. The tRNA sample (0.55 mM) was dissolved in 50 mM sodium cacodylate buffer, pH 6.0, 15 mM MgCl$_2$, 100 mM NaCl, with no EDTA. MnCl$_2$ was then added to give the indicated molar ratios of Mn$^{2+}$/tRNA. (D) the difference spectrum obtained by subtracting the spectrum obtained with 0.2 Mn$^{2+}$/tRNA from that collected in the absence of Mn$^{2+}$.
CHEMICAL SHIFT (PPM FROM FUra)
The polyamine-tRNA interaction is also of interest since polyamines have been found to be essential to grow crystals of tRNA used in x-ray diffraction studies (Ladner et al., 1972; Kim et al., 1974), and two specific spermine binding sites are found in the crystal structure of yeast tRNA\(^{\text{Phe}}\). Determination of the effect of spermine binding on the \(^{19}\text{F}\) NMR spectra of FUra-containing tRNA may aid in assigning the \(^{19}\text{F}\) peaks from FUra residues near the sites of spermine binding.

Addition of one to seven equivalents of spermine results in changes in the chemical shift position of three resonances, A, I, and G, in the \(^{19}\text{F}\) NMR spectrum of (FUra)\(t\text{RNA}^{\text{Val}}\) (Fig. 14). Peak A shifts upfield with increasing amounts of spermine. This shift appears to be a nonspecific ion effect since similar behavior occurs with increasing [NaCl] (Fig. 10) or [NH\(_4\)Cl] (data not shown), or decreasing levels of magnesium (Fig. 9). The resonance originally at 3.6 ppm, peak I, also shifts upfield and merges with peak J upon addition of spermine. Although not affected by magnesium (Fig. 9) or Na\(^+\) (Fig. 10) peak J does shift upfield when NH\(_4\)Cl was added to the tRNA solution (data not shown). The most interesting and most specific effect of spermine on the \(^{19}\text{F}\) NMR spectrum of (FUra)\(t\text{RNA}^{\text{Val}}\) is on a resonance originally at ca. 4.0 ppm, in the G/H region. As increasing amounts of spermine are added, one of the two \(^{19}\text{F}\) resonances in this compound peak shifts progressively downfield and eventually merges with peaks in the D/E/F region. From these results alone, it is not obvious whether this spermine-sensitive \(^{19}\text{F}\) signal is peak G or H; however, tetranucleotide codon (GpUpApA) binding studies have demonstrated that it is peak G that shifts downfield upon spermine addition (see Fig. 29). Peak G shifts only slightly upon removal of magnesium from the tRNA and is completely insensitive to changes in NaCl or NH\(_4\)Cl concentration, indicating that the effect of spermine on this peak is specific.
Figure 14. Effect of spermine on the $^{19}$F NMR spectrum of (FUra)tRNA$_{Val}$. The tRNA (0.32 mM) was dissolved in standard buffer (see Methods) and spermine was added to give the indicated molar ratios of spermine/tRNA.
Temperature dependence of the $^{19}$F NMR spectrum of (FURA)$_n$tRNA$^{Val}$

Previous studies of thermal denaturation of (FURA)$_n$tRNA$^{Val}$ in 100 mM NaCl without Mg$^{++}$ (Hardin, 1984) show that as the temperature is increased, the farthest downfield peaks, B and C (5.5-7.0 ppm), melt first, followed by the upfield peaks (0.5-2.5 ppm), which disappear as the temperature is increased above 70°C. Both groups of resonances shift to the central region of the spectrum, and at 80°C the spectrum collapses into one broad peak centered at 4.7 ppm.

At temperatures below 50°C and in the presence of 15 mM MgCl$_2$, several $^{19}$F resonances shift downfield as the temperature is increased before there is any indication of disruption of the tertiary interactions in the tRNA. The temperature dependence of the $^{19}$F NMR spectrum of (FURA)$_n$tRNA$^{Val}$ in 15 mM Mg$^{++}$ is shown in Figure 15. Peak H is the most temperature sensitive resonance. This peak shifts 0.45 ppm downfield between 17°C and 47°C, ultimately shifting downfield of peak G at 47°C. A resonance in the region between 4.0 and 5.0 ppm (Peaks D-F) also moves downfield with increasing temperature, until at 47°C a well-resolved resonance at 4.9 ppm appears that was not observed at lower temperatures. Although identifying which resonance in the D/E/F region shifts downfield as the temperature increases is difficult, it appears to be the peak labeled F since peaks D and E are clearly visible throughout this temperature range and do not shift. Peak M, originally at 1.9 ppm, also shifts downfield as the temperature is increased, improving the resolution between peaks M and N at higher temperatures.

The line widths of all the $^{19}$F signals in the spectrum narrow with increasing temperature. This effect, coupled with the shifts described above, results in the spectrum obtained at 47°C having resolved $^{19}$F peaks for all 14 fluorouracil residues incorporated in (FURA)$_n$tRNA$^{Val}$.

The changes in the $^{19}$F NMR spectrum of (FURA)$_n$tRNA$^{Val}$ in the presence
Figure 15. Temperature dependence of the $^{19}$F NMR spectrum of (FUra)$_n$ tRNA$^{Val}$. The tRNA (0.38 mM) was dissolved in standard buffer (see Methods) and $^{19}$F NMR spectra were recorded at the temperatures indicated (°C)
84

CHEMICAL SHIFT (PPM FROM FUrA)
of magnesium occur below 50°C, well before melting of the tRNA, as judged by temperature-absorbance studies (Tm = 79°C) (Horowitz et al., 1974; Hardin, 1984). Therefore, these low-temperature changes likely represent a temperature-dependent conformational change in the tRNA structure prior to melting.

**Reaction of FUra-substituted tRNA\textsuperscript{Val} with bisulfite**

Reaction of tRNA with sodium bisulfite at pH 7 provides a specific method for modification of uridine residues in single-stranded regions of the molecule (Furuichi et al., 1970). The product formed, 5,6-dihydrouridine-6-sulfonate, is stable at pH 6, but incubation at pH 9 reverses the reaction. Under similar conditions, 5-fluorouracil also adds bisulfite across the 5,6 double bond, which leads to a large shift of the \(^{19}\text{F}\) signal 35–40 ppm upfield (Sander and Deyrup, 1972). Identification of the \(^{19}\text{F}\) resonances affected by reaction with bisulfite could provide a method for identifying signals from FUra residues in single-stranded regions of the tRNA molecule. Previous studies of bisulfite modification of \((\text{FUra})\text{tRNA}\text{Val}\) (Hardin, 1984) showed that \(^{19}\text{F}\) resonances in the central portion of the spectrum were affected. However, quantification and identification of the reactive residues were not possible in these experiments. The current investigation was undertaken to determine the number and identity of resonances in the \(^{19}\text{F}\) NMR spectrum of \((\text{FUra})\text{tRNA}\text{Val}\) that are affected by reaction with bisulfite.

The \(^{19}\text{F}\) NMR spectrum of bisulfite modified FUra-substituted tRNA\textsuperscript{Val} is shown in Figure 16B. Formation of the bisulfite adduct results in the shift of four \(^{19}\text{F}\) signals from the central portion of the spectrum (3.5–4.5 ppm) upfield to a region 36–37 ppm upfield from free FUra. Comparison with a control spectrum (Fig. 16A), and examination of the differences between the spectra (Fig. 16C) permits identification of the four bisul-
Figure 16. $^{19}$F NMR spectrum of sodium bisulfite modified (FUr)\textsubscript{1}tRNA\textsubscript{\text{Val}}. The reaction with sodium bisulfite was carried out as described in Methods. Spectra were recorded in standard buffer (see Methods). (A) $^{19}$F NMR spectrum of unmodified (FUr)\textsubscript{1}tRNA\textsubscript{\text{Val}} (0.17 mM); (B) spectrum of bisulfite modified (FUr)\textsubscript{1}tRNA\textsubscript{\text{Val}} (0.17 mM); (C) difference spectrum obtained by subtracting (B) from (A)
fite reactive residues as those corresponding to peaks D, E, F, and H, indicating that these residues exist in loop regions of the tRNA.

It is interesting to note that peak G, which is also located in the central portion of the spectrum, does not react with bisulfite under these conditions. Peak G is the only resonance in the central portion of the spectrum that is not pH titratable (Fig. 8); and measurement of the solvent isotope shift (SIS) on transferring the tRNA from H₂O to H²O also indicates that FUra G is not accessible to solvent (Hardin et al., 1986).

Bisulfite modification also leads to a small upfield shift of peak I (Fig. 16B), resulting in peak I coalescing with peak J. The significance of this shift is not clear; it may represent a conformational change in the tRNA due to the chemical modification, or FUra I may be near one of the bisulfite modified FUra residues and experience a change in magnetic environment due to its proximity to the SO₃⁻ adduct.

Valine accepting activity of (FUra)tRNAVal was not affected by bisulfite modification. Both the modified and control sample retained more than 85% of their original activity (data not shown), indicating that bisulfite addition does not produce large conformational changes in the tRNA. Retention of activity in the bisulfite modified tRNA also suggests that the modified FUra residues are not involved in the recognition of this tRNA by valyl tRNA synthetase.

**Photoreaction of 4'-(hydroxymethyl)-4,5',8-trimethyl psoralen with FUra-substituted tRNAVal**

Psoralens are a family of planar aromatic compounds that intercalate in base-paired regions of nucleic acids and can photoreact with the 5,6 double bond of pyrimidines upon irradiation with long-wavelength (350 nm) ultraviolet light (for review see Cimino et al., 1985). Single-stranded nucleic acids also react, but to a lesser extent than helical structures
(Thompson et al., 1982). Bachellerie et al. (1981) have shown uridine is the most reactive base in RNA, and other studies indicate that 5-fluorouracil is even more reactive than uracil (Harter et al., 1974; Ou and Song, 1978). Recently a number of psoralen derivatives, including 4'- (hydroxymethyl)-4,5',8-trimethyl psoralen (HMT), have been developed which are more water soluble and more reactive than the parent compound (Isaacs et al., 1977). These derivatives have been used in a number of studies on RNA structure in solution (Bachellerie and Hearst, 1982; Thompson and Hearst, 1983; Neilsen and Leick, 1985).

Photoaddition of psoralen to FUra residues in FUra-substituted tRNA should perturb the environment of the fluorine of the reacted bases, resulting in a change in the $^{19}\text{F}$ signal from these residues. This method could be used to identify $^{19}\text{F}$ resonances from FUras in double-stranded regions of the tRNA. Figure 17 shows the effects of photoreaction with HMT on the $^{19}\text{F}$ NMR spectrum of (FUra)$_1$tRNA$^\text{Val}$. The largest effects are seen in the upfield portion of the spectrum, between 2.0 and 3.5 ppm. Peaks I, J, and L show a decrease in intensity upon reaction with HMT (compare Fig. 17B with 17A). The furthest downfield resonance, peak A, is also reactive, whereas the peaks in the central portion of the spectrum do not appear to be affected. Two areas of the spectrum have increased signal intensity after reaction with HMT. These regions are located at 4.7 ppm and at ca. 1.6 ppm. These increases may represent a shift of intensity from the signals which are diminished by HMT reaction.

These results indicate that the $^{19}\text{F}$ signals in the upfield portion of the spectrum are derived from FUra residues in structural environments where HMT can intercalate and photoreact, presumably those located in base-paired regions of the tRNA molecule.
Figure 17. $^{19}$F NMR spectrum of (FUra)$_{tRNA^{Val}}$ after reaction with 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT). Reaction with HMT was performed as described under Methods. The spectra were recorded in standard buffer (see Methods). (A) $^{19}$F NMR spectrum of (FUra)$_{tRNA^{Val}}$ (0.31 mM) irradiated at 350 nm in the absence of HMT; (B) the spectrum of HMT modified (FUra)$_{tRNA^{Val}}$ (0.31 mM)
19F NMR Study of Codon-Anticodon Interaction in FUra-Substituted tRNA

The anticodon of FUra-substituted tRNA Val contains two FUra residues (Fig. 2): one at position 34 (the wobble position) normally occupied by uridine-5-oxyacetic acid, and a second at position 33, 5'-adjacent to the anticodon. Binding of complementary oligonucleotides to the anticodon loop is expected to perturb the 19F signals from these FUra residues. To explore the structure of the anticodon loop and assign 19F resonances in the spectrum of (FUra)tRNA Val, interaction of this tRNA with oligonucleotides complementary to the anticodon loop was investigated.

Effect of codon-anticodon interaction on the 19F NMR spectrum of (FUra)tRNA Val

Trinucleotide codons Figure 18 shows the 19F NMR spectra of FUra-substituted tRNA Val recorded in the absence and presence of the codon GpUpA. The only significant change in the spectrum that occurs upon addition of increasing amounts of GpUpA is the progressive upfield shift of the 19F resonance seen at 3.90 ppm in the absence of codon (Peak H, indicated by an asterisk). This behavior indicates that FUra H exists in two different magnetic environments, free and complexed with codon. The shift represents the weighted average of the two forms with rapid exchange between them, i.e., the rate of exchange is greater than πΔν, where Δν is the difference in chemical shift between the free and complexed state expressed in Hertz. In this case Δν is 195 Hz (see below), which means the exchange rate is >> 612 sec⁻¹.

Changes in the position of the peak with increasing concentrations of GpUpA are plotted in Figure 19A. Linear regression analysis of the double reciprocal plot of these data (Fig. 19B) indicates a maximum shift in the resonance position of this peak of 0.69 ppm (195 Hz) at infinite GpUpA concentration, which corresponds to the position of the peak when all the
Figure 18. $^{19}$F NMR spectra of FUra-substituted tRNA$^{\text{Val}}$ before (A) and after addition of 0.43 mM (B) and 2.23 mM (C) G$\text{U}_A$. Solution conditions were 0.33 mM (FUra)tRNA$^{\text{Val}}$ in 50 mM sodium cacodylate buffer, pH 6.0, 100 mM NaCl, 15 mM MgCl$_2$, and 1 mM EDTA. Asterisks indicate peak H in each spectrum.
CHEMICAL SHIFT (PPM FROM FURA)
Figure 19. Analysis of the binding of GpU-A to (FUrA)tRNA\textsubscript{Val}. (A) Plot of the change in chemical shift position, in ppm, of the 19F resonance designated peak H at various concentrations of added GpU-A. Solution conditions were the same as Figure 18 except the concentration of (FUrA)tRNA\textsubscript{Val} was 0.22 mM. (B) Double reciprocal plot of the data in (A). (C) Scatchard plot of GpU-A binding to (FUrA)tRNA\textsubscript{Val}. The ratio of bound to free GpU-A was determined from the fraction of the maximal upfield shift of peak H.
tRNA is complexed with codon. Scatchard analysis of these data, shown in Figure 19C, was used to determine an association constant for the equilibrium:

\[ \text{(FUra)}\text{tRNA}_{\text{Val}}+ \text{GUA} \rightleftharpoons \text{(FUra)}\text{tRNA}_{\text{Val}}:\text{GUA} \]

The calculated binding constant, 2500 M\(^{-1}\) at 25°C and 0.1 M NaCl, agrees well with the value of 3000 M\(^{-1}\) for the association of G\(_p\)U\(_p\)A with normal tRNA\(_{\text{Val}}\), determined by equilibrium dialysis at -2°C and at 1.0 M NaCl (Pongs and Griese, 1972).

Addition of trinucleotides not complementary to the anticodon of tRNA\(_{\text{Val}}\) has no effect on the position of Peak H as shown in Figure 20 for the trinucleotide U\(_p\)A\(_p\)A. Similar results obtained with A\(_p\)A\(_p\)A and A\(_p\)U\(_p\)G (data not shown), indicate that the shift observed with G\(_p\)U\(_p\)A is codon specific and not due to nonspecific effects of added oligonucleotides. A preliminary assignment of the \(^{19}\text{F}\) signal at 3.9 ppm (peak H) to FUra-34 in the anticodon of (FUra)tRNA\(_{\text{Val}}\) can be made on the basis of the results of these trinucleotide codon binding experiments.

**Tetranucleotide codons**

The tetranucleotide G\(_p\)U\(_p\)A\(_p\)A is complementary to the anticodon of FUra-substituted tRNA\(_{\text{Val}}\) and the 5'-adjacent FUra-33; it is expected to have a higher binding constant for the tRNA than G\(_p\)U\(_p\)A (Uhlenbeck, 1972). Addition of G\(_p\)U\(_p\)A\(_p\)A to (FUra)tRNA\(_{\text{Val}}\) produces an upfield shift of the same \(^{19}\text{F}\) resonance, at 3.90 ppm (peak H, indicated with an asterisk), which shifts when G\(_p\)U\(_p\)A is added (Fig. 21). With increasing G\(_p\)U\(_p\)A\(_p\)A concentration, the \(^{19}\text{F}\) signal at 3.90 ppm diminishes in intensity while the area under the peak at 3.23 ppm (indicated by an arrow) increases (Fig. 21); no \(^{19}\text{F}\) resonances are observed at intermediate positions. The position of the shifted peak is that expected from the maximal shift calculated with G\(_p\)U\(_p\)A (0.69 ppm), and at high G\(_p\)U\(_p\)A\(_p\)A concentrations intensity corresponding to approximately one FUra has shifted upfield. This behavior is evidence for a slow exchange
Figure 20. $^{19}$F NMR spectra of FUra-substituted tRNA$^\text{Val}$ before (A) and after (B) addition of 1.95 mM U$_3$ApA. Solution conditions were those of figure 18 except the tRNA concentration was 0.41 mM.
Figure 21. $^{19}$F NMR spectra of FUra-substituted tRNA$^{\text{Val}}_1$ before (A) and after addition of 0.3 mM (B); 1.0 mM (C) and 1.8 mM (D) G$_p$U$_p$A$_p$A. The concentration of tRNA was 0.43 mM in standard buffer (see Methods). Arrow indicates shifted position of peak H.
CHEMICAL SHIFT (PPM FROM FUra)
(<< 612 sec\(^{-1}\)) between the complexed and free forms of the fluorouracil residue; in contrast to the fast exchange observed with \(G_pU_pA\). Such a difference is consistent with greater stability of the complex between the tRNA and \(G_pU_pA\). Scatchard analysis, based on the relative intensities of the two peaks corresponding to the \(^{19}\text{F}\) resonance in the free and complexed state, yields a binding constant of 6800 M\(^{-1}\), which agrees with values found by others for complementary tetranucleotides binding to the anticodons of several tRNAs. For example, Freier and Tinoco (1975) found a value of 6200 M\(^{-1}\) for \(A_pU_pG_pA\) binding to yeast tRNA\(^{\text{Phe}}\) using equilibrium dialysis at 0°C and 1M NaCl. The finding that \(G_pU_pA\) binding to \((\text{FUra})\text{tRNA}^{\text{Val}}\) also affects peak H supports the assignment of this \(^{19}\text{F}\) resonance to FUra-34.

In addition to the upfield shift of Peak H, binding of \(G_pU_pA\) to FUra-substituted tRNA\(^{\text{Val}}\) induces the downfield shift of a peak originally at about 4.5 ppm, in the D/E/F region (Fig. 21). Increasing concentrations of the tetranucleotide cause a progressively larger downfield shift of this resonance (Fig. 21). The affected resonance appears to be peak F, which is originally unresolved, because at high levels of \(G_pU_pA\) the peaks labeled D and E are still visible at their original chemical shift positions. Addition of the tetranucleotide \(A_pU_pG_pA\), which is not complementary to the anticodon loop, causes no significant change in the \(^{19}\text{F}\) NMR spectrum of \((\text{FUra})\text{tRNA}^{\text{Val}}\) (data not shown). Based on the effects of \(G_pU_pA\) binding on the \(^{19}\text{F}\) spectrum of \((\text{FUra})\text{tRNA}^{\text{Val}}\), a preliminary assignment of the \(^{19}\text{F}\) resonance at 4.5 ppm (Peak F) to FUra-33 (5'-adjacent to the anticodon) can be made.

**Characterization of the effect of the 3'-terminal A in \(G_pU_pA\)**

The 3'-terminal adenosine of \(G_pU_pA\) may base-pair directly with FUra-33, and the downfield shift of the \(^{19}\text{F}\) signal at 4.5 ppm (Fig. 21)
may be due to such an interaction. However, for FUra-33 to base-pair with GpUpApA, the anticodon loop would have to undergo a conformational change from the 3'-stacked conformation found in the crystal structure of tRNA^Phe (Fig. 1), to a structure with the anticodon stacked on the 5' side of the loop.

To further characterize the role of the 3'-terminal adenosine in GpUpApA, tetranucleotides which contain the codon GpUpA and a 3'-nucleotide that is not complementary to FUra-33 were constructed, and their effects on the ^19F NMR spectrum of (FUra)tRNA^Val were investigated. GpUpApC and GpUpApU are complementary to the anticodon of tRNA^Val but contain 3'-terminal pyrimidines, which are not complementary to FUra-33. As can be seen in Figure 22, addition of GpUpApU to (FUra)tRNA^Val causes a progressive upfield shift of peak H, at 3.9 ppm, similar to that seen with GpUpA. Peak F (4.5 ppm), however, does not shift downfield as a result of GpUpApU binding to the tRNA (Fig. 22). Similar results were obtained when GpUpApC was used (data not shown), and a binding constant of 3025 M⁻¹ was determined by Scatchard analysis. Figure 23 shows the effect of a 3'-terminal guanosine, which may be capable of forming a G-U base with FUra-33, and should be more efficient than a pyrimidine in stabilizing oligonucleotide binding as a 3'-dangling-end (Westhof et al., 1983). Addition of GpUpApG to (FUra)tRNA^Val again causes peak H to shift upfield (Fig. 23). In this case, the binding has the characteristics of a slow exchange with intensity decreasing at 3.90 ppm while it increases upfield at 3.23 ppm (indicated by an arrow in Fig. 23). Scatchard analysis of this interaction yields a binding constant of ca. 5500 M⁻¹. Again, Peak F at 4.5 ppm does not shift downfield upon addition of GpUpApG (Fig. 23). These findings support the view that the effect of GpUpApA on the ^19F resonance at 4.5 ppm in the spectrum of (FUra)tRNA^Val is not simply due to the presence of a 3'-dangling end on GpUpA, but is specific for the
Figure 22. $^{19}$F NMR spectra of (FUra)$_7$tRNA$_{Val}^1$ before (A) and after addition of 0.3 mM (B); 0.6 mM (C); and 1.0 mM (D) GpUpApU. Spectra were recorded in standard buffer (see Methods). The concentration of (FUra)$_7$tRNA$_{Val}^1$ was 0.17 mM. Asterisks indicate the position of peak H in each spectrum.
Figure 23. $^{19}\text{F}$ NMR spectra of FUra-substituted $\text{tRNA}^{\text{Val}}$ before (A) and after addition of 0.26 mM (B); 0.78 mM (C); and 1.3 mM (D) G$_p$U$_p$A$_p$G. Spectra were recorded in standard buffer (see Methods). The concentration of (FUra)$\text{tRNA}^{\text{Val}}$ was 0.35 mM. Arrow indicates shifted position of peak H.
CHEMICAL SHIFT (PPM FROM FUr a)
3'-terminal adenosine, which is complementary to FUra-33.

**Formation of an anticodon-anticodon complex**

The anticodons of *E. coli* tRNA$^\text{lyr}$ (anticodon QUA, where Q is a modified guanosine) and FUra-substituted tRNA$^{\text{Val}}$ (anticodon FAC) are complementary and can form a highly stable complex by hydrogen bonding between their anticodons (Eisinger, 1971). The binding constants for such anticodon-anticodon complexes are several orders of magnitude greater than those for oligonucleotides binding to the anticodon loop, and this interaction has been used as a model for codon-anticodon interaction on the ribosome (Grosjean et al., 1976). Addition of an equimolar amount of tRNA$^\text{lyr}$ to (FUra)tRNA$^{\text{Val}}$ causes peak H at 3.9 ppm to shift upfield to ca. 3.2 ppm (Fig. 24). The observed shift agrees with the maximum shift determined for GpUpA binding, and is the result expected for a tight codon-anticodon interaction. This result again supports the assignment of peak H to FUra-34 in the anticodon of (FUra)tRNA$^{\text{Val}}$.

Significant broadening of all the resonances in the $^{19}$F spectrum of the complex was observed, which is consistent with a decrease in rotational correlation due to the increased molecular weight of the tRNA:tRNA complex. The $^{19}$F signal at 4.5 ppm does not shift downfield upon formation of the anticodon-anticodon complex, showing that this shift cannot be ascribed simply to a strong association between the anticodon and a complementary base sequence.

**Evidence that codon-containing oligonucleotides bind to the anticodon loop**

1H NMR To confirm that the effects of codon-containing oligonucleotides are caused by binding to the anticodon of (FUra)tRNA$^{\text{Val}}$, 1H NMR studies of the methyl proton resonances in the tRNA were undertaken. (FUra)tRNA$^{\text{Val}}$ contains m$^6$A at position 37, 3'-adjacent to the anticodon. Codon binding to the anticodon should perturb the proton resonances of the
Figure 24. $^{19}$F NMR spectra of FURA-substituted tRNA$^{Val}_1$ recorded before (A) and after complex formation with E. coli tRNA$^{Yr}_2$ (B). Spectra were recorded in standard buffer, and the concentrations of the tRNAs was 0.3 mM each.
methyl group. Geerdes et al. (1980a) showed that binding of the codon-containing oligonucleotide $U_pU_pC_pA$ to tRNA$^{\text{Phe}}$ of yeast or E. coli produces upfield shifts of the methyl resonances of the hypermodified base adjacent to the anticodon. The methyl region $^1$H NMR spectrum of (FUr)RNA$^\text{Val}$ contains proton resonances derived from the m$^7$G at position 46 and the m$^3$A in the anticodon loop. The methyl protons from these residues in tRNA$^\text{Val}$ have been assigned by Kastrup and Schmidt (1975, 1978) to resonances 3.82 ppm (m$^7$G) and 2.50 ppm (m$^6$A) downfield from DSS in $^1$H NMR spectra recorded at 27°C in 10 mM sodium phosphate pH 7.0. Figure 25 shows the $^1$H NMR spectra of the methyl protons of (FUr)RNA$^\text{Val}$ at 25°C in 30 mM sodium phosphate pH 6.0 before and after addition of G$_pU_pA_pA$. Upon addition of increasing amounts of oligonucleotide, the resonance originally at 2.48 ppm shifts progressively upfield. Similar results are obtained with G$_pU_pA$, but addition of A$_pU_pG_pA$, which is not complementary to the anticodon has no affect on the peak at 2.48 ppm (data not shown). The $^1$H signal from the methyl group of m$^7$G (3.80 ppm) was not affected by the addition of either G$_pU_pA_pA$ or A$_pU_pG_pA$. Perturbation of the $^1$H resonance from the m$^6$A methyl protons by G$_pU_pA$ and G$_pU_pA_pA$ indicates that these oligonucleotides are binding to the anticodon.

Cleavage of dG$_pT_pA_pA$: (FUr)RNA$^\text{Val}$ by ribonuclease H

Ribonuclease H is an endonuclease that cleaves the RNA portion of DNA:RNA hybrids. This enzyme has been used to study the solution structure of a number of RNAs by determining which portions of the molecule are accessible to complementary deoxyoligonucleotides binding, as measured by RNase H cleavage (Harris, 1979; Mankin et al., 1981; Reitewld et al., 1982). Binding of the deoxyoligonucleotide dG$_pT_pA_pA$ to (FUr)RNA$^\text{Val}$ produces an upfield shift of the same $^1$H peak at 3.9 ppm (peak H) as does G$_pU_pA$ and G$_pU_pA_pA$ binding (Fig. 26). This result indicates that the deoxyoligonucleotide
Figure 25. 300 MHz $^1$H NMR spectra of $m^6$A methyl protons in FUra-substituted tRNA$^{\text{I}}_{\text{Al}}$ (0.58 mM) before (A) and after addition of 0.28 mM (B) and 0.84 mM (C) GpUpApA. Conditions were 30 mM sodium phosphate, pH 6.0, 20 mM MgCl$_2$, and 100 mM NaCl in 100% $^2$H$_2$O. Asterisks indicate impurities.
Figure 26. $^{19}\text{F} \text{NMR}$ spectra of $(\text{FUra})t\text{RNA}^\text{Val}_1$ before (A) and after addition of 0.9 mM (B) and 2.7 mM (C) $dGpTpApA$. Spectra were recorded in standard buffer (see Methods). The tRNA concentration was 0.38 mM
binds to the same site on (FUra)tRNA$\text{Val}$ as the codon-containing oligoribonucleotides. Figure 27 shows that when the complex of the deoxyoligonucleotide and 5'-$^{32}$P end-labeled (FUra)tRNA$\text{Val}$, formed under a variety of conditions, is hydrolyzed with RNase H, cleavage occurs at one major site, position 34 in the anticodon loop. This indicates that dGpTpApA binds only to the anticodon loop. It is, therefore, reasonable to conclude that GpUpA and GpUpApA also bind only to the anticodon loop.

Influence of magnesium and spermine on codon-anticodon interaction

Both magnesium and spermine serve to stabilize the conformation of the anticodon arm in the crystal structure of yeast tRNA$\text{Phe}$ (Quigley et al., 1978). One spermine molecule is bound at the junction of the D and anticodon stems. The presence of this polycation has a noticeable effect on the conformation of the anticodon stem, producing a $26^\circ$ kink in the double helix between the anticodon and D stems. The anticodon conformation is further stabilized by a magnesium ion bound to the upper part of the loop. Together these ligands significantly immobilize the anticodon loop (Teeter et al., 1980).

The previous studies of oligonucleotide binding to the anticodon of (FUra)tRNA$\text{Val}$ were done in the presence of 15 mM Mg$^{++}$ (no spermine). To determine the influence of magnesium and spermine on the codon-anticodon interaction, oligonucleotide codon binding to (FUra)tRNA$\text{Val}$ was examined in the absence of Mg$^{++}$, and in the presence of both spermine and magnesium. Addition of the codon GpUpA to (FUra)tRNA$\text{Val}$ in the absence of Mg$^{++}$, results in the upfield shift of $^{19}F$ resonances, at 3.29 and 3.89 ppm (Fig. 28). The chemical shift positions of these signals indicate they are peaks H and F, respectively (Fig. 9B). These are the same $^{19}F$ resonances affected by codon binding in the presence of 15 mM Mg$^{++}$ (Figs. 18 and 21). Both peaks shift upfield continuously as oligonucleotide is
Figure 27. Ribonuclease H cleavage of 5'-32P end-labeled (FUra)trNA Val Standard reactions mixtures contained 0.5 pMol tRNA, 50 pmol dG₃p₅T₃p₃ApA in 40 mM Tris-HCl, pH 7.9, 8 mM MgCl₂, 2 mM DTT, and 30 µg/mL BSA. All reactions were incubated at 30°C for 30 min with 0.5 units RNase H (unless otherwise indicated). Lane 1: preincubated 30 min at 30°C without enzyme prior to start of reaction. Lane 2: reaction without preincubation. Lane 3: 100 mM NaCl added to the reaction mixture. Lane 4: reaction in NMR buffer: 50 mM sodium cacodylate buffer, pH 7.9, 15 mM MgCl₂, 100 mM NaCl, 30 µg/µL BSA. Lane 5: Ribonuclease T1 sequencing ladder. Lane 6: reaction lacking dG₃p₅T₃p₃ApA. Lane 7: reaction without RNase H.
Figure 28. Effect of GpUpA on the $^{19}$F NMR spectrum of (FURA)$_n$ in the absence of magnesium. Spectra were recorded before (A) and after addition of 0.50 mM (B); 1.0 mM (C); and 2.0 mM (D) GpUpA. Solution conditions were 50 mM sodium cacodylate buffer, pH 6.0, 100 mM NaCl, and 1 mM EDTA. The concentration of tRNA was 0.32 mM
CHEMICAL SHIFT [PPM FROM FUrA]
added; however, peak H shifts nearly twice as far as peak F. Eventually, at 2 mM \text{GpUpA}, peak H has shifted 0.28 ppm while peak F has moved only 0.15 ppm.

When the tetranucleotide \text{GpUpA} is added to (FUra)tRNA$_{Val}$ in the absence of Mg$^{++}$, the same two resonances again shift progressively upfield (data not shown). In this case, however, the magnitude of the shifts are approximately the same for each peak, with a maximum shift of nearly 0.5 ppm upon addition of 1.4 mM \text{GpUpA}.

These results contrast with those observed for tri- and tetranucleotide codon binding to (FUra)tRNA$_{Val}$ in 15 mM Mg$^{++}$ (Figs. 19 and 21). In the presence of Mg$^{++}$, \text{GpUpA} binding induces the upfield shift of only peak H, whereas \text{GpUpA} induces peak F to shift downfield in addition to the upfield shift of peak H. The association constants for \text{GpUpA} and \text{GpUpApA} binding to (FUra)tRNA$_{Val}$ are much lower in the absence of Mg$^{++}$; both are estimated at ca. 300 M$^{-1}$ by Scatchard analysis (data not shown). The results obtained in the absence of magnesium are consistent with an alternate anticodon loop conformation in the absence of the cation (see Discussion).

Figure 29 shows the effect of \text{GpUpApA} binding on the $^{19}$F NMR spectrum of (FUra)tRNA$_{Val}$ in the presence of spermine (and 15 mM Mg$^{++}$). Increasing amounts of \text{GpUpApA} shift peak H (3.8 ppm) upfield to 3.16 ppm, which again agrees with the maximum of shift peak H, 0.69 ppm, calculated for \text{GpUpA} binding. Peak F (4.5 ppm) does not shift downfield as occurs upon \text{GpUpApA} binding to (FUra)tRNA$_{Val}$ in 15 mM Mg$^{++}$ without spermine (Fig. 21). It is possible that spermine stabilizes a conformation of the anticodon loop in which the 3'-terminal adenosine of \text{GpUpA} cannot interact with FUra-33.

Addition of spermine to FUra-substituted tRNA$_{Val}$ in the presence of MgCl$_2$ (15 mM) was shown to cause a downfield shift of one $^{19}$F resonance in the G/H region (Fig. 15). The peak that shifted was difficult to identify
Figure 29. Effect of G\textsubscript{p}U\textsubscript{p}A\textsubscript{p}A on the $^{19}$F NMR spectrum of (F\textsubscript{U}ra)tRNA\textsubscript{Val} in the presence of spermine. Spectra were recorded before (A) and after addition of 0.85 mM (B); 1.7 mM (C); and 3.4 mM (D) G\textsubscript{p}U\textsubscript{p}A\textsubscript{p}A. The tRNA (0.32 mM) was dissolved in standard buffer (including 15 mM Mg\textsuperscript{2+}) with 2.10 mM spermine.
because peaks G and H overlap. The upfield shift of the peak at 3.8 ppm upon addition of $G_pU_pA_pA$ in the presence of spermine (Fig. 29) indicates that this is peak H and that peak G is the resonance shifted downfield by spermine (Fig. 15).

**Codon-anticodon interaction in FUra-substituted methionine tRNAs**

FUra-substituted methionine tRNAs also contain a FUra residue in their anticodon. In this case, the FUra is located in the 3' position of the anticodon as opposed its 5' location in (FUra)$tRNAm^\text{Val}$. $^{19}$F NMR studies of codon binding to FUra-substituted tRNA$^\text{Met}_m$ show that one $^{19}$F resonance, originally at 4.2 ppm (peak I), shifts progressively upfield upon addition of increasing amounts of the codon $A_pU_pG$ (Fig. 30). Analysis of a double reciprocal plot of the binding curve indicates 0.16 ppm as the maximum shift in the position of peak I at infinite $[A_pU_pG]$, and Scatchard analysis of these data yields a binding constant of 1200 $M^{-1}$ at 20°C and 0.1 M NaCl (data not shown but similar to Fig. 19). The calculated binding constant agrees well with the previously reported value of 1700 $M^{-1}$ for this interaction, as determined by equilibrium dialysis at 2°C and 0.2 M NaCl (Hogenauer et al., 1972). Addition of $G_pU_pA_pA$ did not effect this peak (data not shown). The chemical shift of peak I (4.2 ppm) is similar to the position of peak H (3.9 ppm) in the $^{19}$F NMR spectrum of (FUra)$tRNAm^\text{Val}$ which, also shifts upfield upon addition of codon (Fig. 18).

In contrast to the results seen with tRNA$^\text{Met}_m$, addition of the codon $A_pU_pG$ has no effect on the $^{19}$F NMR spectrum of 5-fluorouracil-substituted tRNA$^\text{Met}_F$ (Fig. 31), even when the codon is added in 6-fold molar excess. Addition of the codon-containing tetranucleotide $A_pU_pG_pA$ also has no effect on the $^{19}$F spectrum of (FUra)$tRNAm^\text{Met}_F$ (results not shown). These results indicate that (FUra)$tRNAm^\text{Met}_F$ either does not bind the oligonucleotide codons, or that the interaction does not result in a visible change
Figure 30. $^{19}$F NMR spectra of FUra-substituted tRNA$_{\text{Met}}$ recorded before (A) and after addition of 0.84 mM (B); 1.68 mM (C); and 3.4 mM (D) A$_p$U$_p$G. Spectra were recorded in standard buffer. The concentration of (FUra)tRNA$_{\text{Met}}$ was 0.43 mM.
CHEMICAL SHIFT (PPM FROM Fura)
Figure 31. $^{19}$F NMR spectra of FUra-substituted tRNA$^{Met}$ recorded before (A) and after addition of 0.90 mM (B) and 1.8 mM ApUpG. Spectra were recorded in standard buffer. The concentration of (FUra)tRNA$^{Met}$ was 0.48 mM.
in the spectrum.

\[ ^{19}F \text{ NMR study of possible conformational change resulting from codon-anticodon interaction} \]

Conformational changes in distant parts of the tRNA molecule have been attributed to association of codon with the anticodon. In particular, it has been suggested that the interactions between the T and D loops are disrupted upon codon binding (Schwarz et al., 1976; Schwarz and Gassen, 1977).

In 5-fluorouracil-substituted tRNA\(^{\text{Val}}\) the T\(^{p}p\text{cp}G\) sequence is replaced by F\(^{p}p\text{cp}G\) since FUra replaces ribothymidine and pseudouridine. If this sequence is exposed then binding of \(C_p\text{gp}A\text{p}A\) should perturb the \(^{19}F\) resonances from these FUras in the spectrum of (FUra)tRNA\(^{\text{Val}}\), much as binding of codon-containing oligonucleotides affects \(^{19}F\) signals from FUra residues in the anticodon loop (Figs. 18, 21 and 30). Figure 32 shows the \(^{19}F\) NMR spectrum of (FUra)tRNA\(^{\text{Val}}\) in the absence (A) and presence (B) of \(C_p\text{gp}A\text{p}A\), and after addition of both \(C_p\text{gp}A\text{p}A\) and G\(^{p}U\text{p}A\) (C). No significant changes in the spectrum occur upon addition of \(C_p\text{gp}A\text{p}A\) alone, and in the presence of both codon and \(C_p\text{gp}A\text{p}A\) the only change is the upfield shift of the \(^{19}F\) resonance originally at 3.90 ppm, which is the same effect seen in Figure 18 for addition of G\(^{p}U\text{p}A\) alone. These results indicate that the F\(^{p}p\text{cp}G\) sequence in the T loop of (FUra)tRNA\(^{\text{Val}}\) is not available for binding to a complementary oligonucleotide, and that codon binding does not induce a conformational change which makes this sequence available for oligonucleotide binding.
Figure 32. $^{19}$F NMR spectra of (FUr)\textsubscript{tRNA}\textsuperscript{Val} recorded before (A) and after addition of 2 mM C\textsubscript{p}G\textsubscript{p}A\textsubscript{p}A (B) and 2 mM C\textsubscript{p}G\textsubscript{p}A\textsubscript{p}A plus 3.5 mM G\textsubscript{p}U\textsubscript{p}A. Spectra were recorded in standard buffer. The concentration of tRNA was 0.66 mM.
CHEMICAL SHIFT (PPM FROM FUra)
DISCUSSION

_E. coli_ tRNA\textsuperscript{Met}_m is the third 5-fluorouracil-substituted transfer RNA to be purified and its $^{19}$F NMR spectrum obtained. Like the first two, tRNA\textsuperscript{Val}_1 (Horowitz et al., 1974) and tRNA\textsuperscript{Met}_f (Hills et al., 1983), this analogue substituted molecule, with over 90% of its uracil and uracil-derived constituents replaced by FUra (Table 3), appears to be fully recognized by its cognate synthetase and aminoacylated to an extent comparable to normal tRNA\textsuperscript{Met}_m. Thus, these analogue substituted tRNAs present useful systems for investigation by $^{19}$F NMR.

The $^{19}$F NMR spectrum of (FUra)tRNA\textsuperscript{Met}_m (Figure 6) contains 13 well resolved resonances and several shoulders derived from the 18 fluoro-uracils in the molecule. The range of chemical shifts for the $^{19}$F resonances in the spectrum of (FUra)tRNA\textsuperscript{Met}_m, from 0.3 to 8.0 ppm downfield from free FUra (= 0 ppm), is similar to that seen in the spectra of FUra-substituted tRNA\textsuperscript{Val}_1 (Fig. 3A) and tRNA\textsuperscript{Met}_f (Fig. 3B). This dispersion of the $^{19}$F signals has been shown to be due to the folding of the tRNA molecule into its native three-dimensional structure. When the secondary and tertiary structure of (FUra)tRNA\textsuperscript{Val}_1 are disrupted, the $^{19}$F NMR spectrum collapses into a single peak at 4.7 ppm, corresponding to the central portion of the native spectrum (Hardin et al., 1986). The $^{19}$F NMR spectrum of poly(FU) (random coil) also yields a single peak at 4.5 ppm (Hardin et al., 1986). These observations indicate that the chemical shifts of the $^{19}$F resonances in the spectra of native FUra-substituted tRNA reflect the different environments of each FUra residue, due to the structure of the folded molecule.

The overall appearance of the spectrum of (FUra)tRNA\textsuperscript{Met}_m (Fig. 6) is also similar to that of FUra-substituted tRNA\textsuperscript{Val}_1 (Fig. 3A) and of tRNA\textsuperscript{Met}_f (Fig. 3B). In each spectrum, there is a group of peaks between 4.0 and
5.0 ppm, close to the chemical shift position of heat denatured (FUra)tRNA and poly(FU) (random coil). These signals presumably correspond to FUra residues in relatively unstructured regions of the native tRNA molecule, i.e., nonbase-paired FUra residues. The $^{19}$F spectra of all three tRNAs also contain a group of peaks upfield of the central region (ca. 0.5-3.8 ppm), and several resolved resonances downfield of 5.0 ppm. It has been proposed (Horowitz et al., 1977) that the downfield resonances represent FUra residues involved in tertiary interactions, and that the group of signals in the upfield portion of the spectra correspond to FUra residues in helical environments.

A comparison of the number of FUra residues in each environmental classification in each tRNA, based on analogy to the crystal structure of tRNA$^{\text{Phe}}$ (Rich and RajBhandary, 1976), and the relative areas of the $^{19}$F signals in the various regions of the spectra, shows a good correlation between structure and chemical shift for FUra-substituted tRNA$^{\text{Val}}_1$ and tRNA$^{\text{Met}}_f$. Both molecules contain three FUra residues involved in tertiary interactions (Fig. 2), and both $^{19}$F spectra contain three peaks in the downfield region, between ca. 5.5 and 8.0 ppm (Fig. 3). The $^{19}$F spectra tRNA$^{\text{Val}}_1$ and tRNA$^{\text{Met}}_f$ show 6, and 3 signals upfield of the central group of peaks, respectively, and this corresponds to the number of FUra residues in helical regions in each of these fluorinated tRNAs. The number of $^{19}$F signals in the central group of peaks in the spectra of (FUra)tRNA$^{\text{Val}}_1$ and (FUra)tRNA$^{\text{Met}}_f$ also agrees with the number of non-hydrogen-bonded FUra residues in each tRNA.

In the case of FUra-substituted tRNA$^{\text{Met}}_m$, the groupings do not appear to correlate as well. The downfield region of the spectrum contains peaks A-D with a total area of four fluorines (Fig. 6), but there are only three FUra residues involved in tertiary interactions in this molecule (Fig. 2). There are 7 secondary base-paired, and 8 non-hydrogen-bonded FUra residues.
in (FUra)tRNA_{m}^{\text{Met}} (Fig. 2), and these numbers do not correlate well with the number of resonances in the upfield and central regions of the $^{19}$F spectrum, respectively (Fig. 6). This indicates that factors other than involvement of the FUra residues in primary, secondary, or tertiary structures are involved in determining the chemical shift positions of $^{19}$F peaks from FUra residues in tRNA. It should be pointed out that few studies have been done with FUra-substituted tRNA_{m}^{\text{Met}}, and more investigation is required before the significance of the grouping of peaks in the $^{19}$F spectrum of this tRNA can be understood.

All three purified FUra-substituted tRNAs can be resolved into two, fully active, isoaccepting species termed the A and B forms of each analogue substituted tRNA. $^{19}$F NMR spectra of the A and B forms of each tRNA differ mainly by the shift of one peak from between 4.6 and 4.8 ppm in the spectrum of isoacceptor B to ca. -15 ppm in the spectra of form A (Figs. 2 and 6). The $^{19}$F signal at -15 ppm has been shown to be due to a ring-opened 5-fluoro-5,6-dihydrouridine substituent present in the A form of these tRNAs (Horowitz et al., 1983).

The modified FUra has been located at position 20 in (FUra)tRNA_{m}^{\text{Met}} by sequence analysis of the two isoacceptors (Hills et al., 1983), and on this basis, a resonance at 4.8 ppm (Peak E) in the $^{19}$F NMR spectrum of (FUra)tRNA_{m}^{\text{Met}} form B (Fig. 3B) has been assigned to FUra 20, normally occupied by the only dihydrouridine in tRNA_{m}^{\text{Met}}. Aniline cleavage at pH 4.5 has located the modified fluorodihydrouridine in FUra-substituted tRNA_{1}^{\text{Val}} at position 17 (Fig. 7). This allows assignment of peak D in the $^{19}$F NMR spectrum of (FUra)tRNA_{1}^{\text{Val}} form B to FUra-17 in the D loop of the tRNA. This position is also the site of the only dihydrouridine in this molecule. E. coli tRNA_{m}^{\text{Met}} normally contains dihydrouridine residues at positions 17, 20, and 20a, and a partial modification at position 16 (Cory and Marker, 1970). Aniline treatment of the A form of 5'-$^{32}$P end-labeled
(FUra)\textsubscript{\text{Met}} results in only one major cleavage in the D loop, at position 20, indicating only one modified fluorodihydrouridine occurs in this tRNA (at position 20). It is, therefore, possible to assign the $^{19}\text{F}$ resonance at 4.6 ppm (peak F) to FUra-20 in (FUra)\textsubscript{\text{Met}}. The results of aniline treatment of clearly show that positions 16 and 17 of (FUra)\textsubscript{\text{Met}}, which normally contain dihydrouridine, are not reduced to fluorodihydrouridine. Modification at position 20a cannot be completely ruled out because the tRNA was labeled at the 5' end. However, since the aniline cleavage conditions used, resulted in only partial cleavage of the polynucleotide backbone, it is unlikely that position 20a is modified. Evidently, the enzyme responsible for dihydrouridine synthesis only recognizes FUra in position 20 of (FUra)\textsubscript{\text{Met}}. This may mean that more than one enzyme catalyzes formation of dihydrouridine in \textit{E. coli} tRNAs, and only one of these enzymes can recognize FUra.

Examination of the Effects of Solution Conditions and Chemical Reactivity of the $^{19}\text{F}$ Resonances in the Spectra of FUra-Substituted tRNA

Examination of the effects of solution conditions and chemical reactivity of $^{19}\text{F}$ resonances in the spectra of purified FUra-substituted tRNAs provides an initial approach to assigning the $^{19}\text{F}$ peaks in the spectra to individual FUra residues in the molecules. Eventually, these studies may also yield valuable information regarding the conformational states of tRNA solution.

Because $^{19}\text{F}$ NMR spectra of heat denatured (FUra)tRNA, and poly(FU) (random coil) show a single peak at ca. 4.7 ppm (Hardin et al., 1986), it has been suggested that the groups of peaks in the central portion (3.9-5.0 ppm) of the $^{19}\text{F}$ NMR spectra of (FUra)tRNAs represent FUra residues in relatively unstructured (loop) regions of the native tRNA molecule.
Several studies presented here support this assignment.

The pH dependence of the \(^{19}\text{F}\) NMR spectrum of (FUra)\(_{tRNA}^{\text{Val}}\) (Fig. 8) shows that FUra residues D, E, F, and H are susceptible to titration and exhibit pKas near that of free FUrd, indicating that these FUra residues are exposed to solvent. The chemical shift of one peak in the central portion of the spectrum (peak G) is, however, unaffected by changes in pH.

Two resonances in the spectrum shift in the acid pH range. Peak C, shifts downfield, and one peak, at 4.5 ppm (labeled F) shifts upfield at pHs between 4.5 and 5.5 (Fig. 8B). Peak C at 5.5 ppm, also shifts downfield with increasing pH, but the extent of this shift is not as great as that of the other peaks, and several other studies, including measurement of the solvent isotope shift (SIS) (Hardin et al., 1986) and reaction with bisulfite (see below) indicate FUra C is not exposed to solvent. These findings suggest that the downfield movement of peak C with increasing pH is not due to ionization of FUra C. Peaks E and F merge between pH 5.5-6.0, and this makes it difficult to identify which of the two shifts at low pH. However, addition of G\(_{p}U_{p}A_{p}A\) to (FUra)\(_{tRNA}^{\text{Val}}\) at pH 4.5 shifts the resonance at 5.2 ppm, which indicates that it is peak F which is affected by low pH (data not shown). Several studies have shown that a structural change occurs in tRNA at mildly acidic pH (Bina-Stein and Crothers, 1975; Steinmetz-Kayne et al., 1977). The shift of peak C, and especially the downfield movement of the peak F (Fig. 8) may reflect this structural change. Peak F has been assigned to FUra 33, and this suggests that the structure of the anticodon loop is affected by acidic pH.

The same FUra residues, those corresponding to peaks D, E, F, and H, in the central portion of the spectrum of (FUra)\(_{tRNA}^{\text{Val}}\) that are susceptible to pH titration, react with bisulfite, a single-strand specific reagent (Fig. 16). This finding is a further indication that these FUra residues are located in nonbase-paired regions of the tRNA. Again, peak G
is an exception, being the only resonance in the 3.9 to 5.0 ppm range that is not affected by bisulfite reaction. Measurement of the solvent isotope shift (SIS) on transfer of the tRNA from H₂O to ²H₂O also shows that FUrA residues D, E, F, and H are completely exposed to solvent, whereas FUrA G appears to have little or no contact with the solvent (Hardin et al., 1986).

FUrA-substituted tRNA<sub>Val</sub> contains 5 non-base-paired FUrA residues (Fig. 2). The results of pH titration (Fig. 8) and reaction with bisulfite (Fig. 16), along with SIS measurements (Hardin et al., 1986) allow assignment of four of these residues to peaks D, E, F, and H, all in the central region of the ¹⁹F spectrum. Peak D has been assigned to FUrA 17 in the D loop based on comparison of the two isoaccepting forms of (FUrA)<sub>Val</sub> (Fig. 7), and peaks F and H have been assigned to the two FUrA residues in the anticodon loop based on oligonucleotide binding studies (Figs. 18 and 21; also see later Discussion). All of these assigned FUrA residues are located in single-stranded regions of the tRNA. There are two more non-hydrogen-bonded FUrA residues in (FUrA)<sub>Val</sub> and only one more fluorouracil, FUrA E, with single-stranded characteristics, i.e., susceptible to pH titration, bisulfite reactive and showing a high SIS. Peak G is also found in the central group of resonances in the ¹⁹F spectrum, suggesting that FUrA G may be the fifth single-stranded FUrA in the tRNA. However, FUrA G is not titratable (Fig. 8) nor does it react with bisulfite (Fig. 16). A possible candidate for FUrA G is position 59 in the T loop. This base is in a single-stranded region of the tRNA, but a number of chemical modification studies show that this position has little reactivity with a variety of chemical reagents (reviewed by Goddard, 1977), and that in general, the T loop is buried in the tertiary structure of the molecule (Vlassov et al., 1981; Romby et al., 1985).

Studies of spermine binding to (FUrA)<sub>Val</sub> show that peak G is specifi-
cally affected by the polyamine (Fig. 14), which suggests that FUra G may be located at position 8, based on comparison to \(^1\)H NMR studies of spermine binding to tRNA (Hyde and Reid, 1985; also see later Discussion). Clearly further investigation is required to make any assignment of peak G, and this assignment will be quite interesting considering the unusual behavior displayed by this \(^{19}\)F signal.

The \(^{19}\)F spectra of all three purified FUra-containing tRNAs contain several resolved resonances downfield (5.5-8.0 ppm) from the central cluster of peaks. Based on the relative areas of the downfield resonances in \(^{19}\)F spectra of both unfraccionated FUra-containing tRNA and of \((\text{FUra})\text{tRNA}^\text{Val}\), Horowitz et al. (1977) suggested that these downfield resonances correspond to FUra residues involved in tertiary interactions in the tRNA molecule. Several recent observations support this hypothesis.

The downfield resonances are the first to melt as the temperature is raised (Hardin, 1984), and therefore, are likely to represent FUra residues involved in tertiary hydrogen-bonding since the tertiary structure has been shown to be the most labile in some tRNAs (Crothers and Cole, 1978). However, interpretation of these results is complicated since recent \(^1\)H NMR studies that shown that in some tRNAs the tertiary interactions are not always the most labile (Johnson and Redfield, 1981; Roy and Redfield, 1983).

\(^{19}\)F\(^{19}\)F nuclear Overhauser studies have provided evidence that the two farthest downfield peaks in the \(^{19}\)F NMR spectrum of \((\text{FUra})\text{tRNA}^\text{Val}\) represent FUra 54 and FUra 55 in the T loop (Hardin, 1984); both of these bases are involved in tertiary interactions (Fig. 2). These studies show a reciprocal NOE between peaks A and B, and based on the structure of yeast tRNA\(^\text{Phe}\), FUra 54 and FUra 55 are the only two FUra residues close enough to each other (4-5 \(\text{Å}\)) to allow an appreciable \(^{19}\)F homonuclear NOE.

Evidence for assignment of peak B to FUra 54, which replaces the
invariant ribothymidine in the T loop, has been provided by $^{19}$F NMR studies of thermal denaturation of (FUra)\textsubscript{tRNA}\textsubscript{Val}, in the absence of Mg\textsuperscript{2+} (Hardin, 1984). As the temperature is increased above 30°C, peak B gradually shifts from 6.6 to 6.4 ppm. Peak B is split into two signals during this transition indicating that FUra B exists in two distinct states that exchange slowly. This behavior resembles splitting observed in the methyl proton resonance of rT 54 in native tRNA\textsubscript{Val} (Kastrup and Schmidt, 1975, 1978; Davanloo et al., 1979) and in the $^{13}$C-methyl signal of rT 54 in several tRNAs (Kopper et al., 1983). Based on this similarity, peak B in the $^{19}$F spectrum of (FUra)\textsubscript{tRNA}\textsubscript{Val} has been tentatively assigned to FUra 54 (Hardin, 1984). A preliminary assignment of peak A to FUra 55 can also be made, based on the $^{19}$F[$^{19}$F] NOE studies mentioned earlier.

$^{19}$F NMR spectra of FUra-substituted tRNA also contain a group of resonances located upfield of the central cluster of peaks (0.5-3.5 ppm). Several lines of evidence support the hypothesis that these resonances correspond to FUra bases in helical structures in the tRNA.

The primary effect of HMT photoaddition to (FUra)\textsubscript{tRNA}\textsubscript{Val} is reduction of the intensity of peaks J and L, in the upfield region of the $^{19}$F spectrum (Fig. 17). Because psoralens are known to react preferentially with pyrimidines in helical structures (Cimino et al., 1985), this result suggests that the FUra bases corresponding to the upfield peaks are located in base-paired stems of the tRNA. The intensity of peak A also decreases substantially upon photoreaction with HMT (Fig. 17). This resonance has been tentatively assigned to FUra 55 in the T loop. Pseudouridine 55, and several of the adjacent bases, are involved in tertiary base-pairs (Fig. 1B). It is possible that these tertiary interactions create an environment which allows the psoralen to intercalate and photoreact with FUra 55. This position is not reactive with HMT in E. coli tRNA\textsubscript{Phe} (Bachellerie and Hearst, 1982), however, pseudouridine in position 55 of
this tRNA is not reactive with psoralen, whereas 5-fluorouracil in the modified tRNA is photoreactive.

Additional evidence to support the assignment of the upfield peaks to FUra residues in helical environments is provided by the observation that ethidium bromide specifically affects resonances L and N at 2.5 and 1.9 ppm, respectively, in the $^{19}$F spectrum of (FUra)$_t$RNA$_{Val}$ (W. C. Chu and J. Horowitz, Dept. of Biochemistry and Biophysics, Iowa State Univ. unpublished results). $^1$H NMR studies have shown that the imino proton from the A6-U67 base-pair in the acceptor stem of tRNA$_{Val}$ is the most sensitive to interaction with ethidium bromide (B. Reid, Dept. of Chemistry, Univ. of Washington, personal communication).

The results discussed in this section have allowed a correlation to be drawn between the various regions of the $^{19}$F NMR spectrum of 5-fluorouracil-substituted tRNAs and the FUra residues in different environments in the tRNA. This will allow general interpretation of effects on the $^{19}$F spectra of these tRNAs and will pave the way for future experiments to make more specific assignments.

Ion binding to FUra-substituted tRNA

The resonances in the $^{19}$F NMR spectra of purified FUra-substituted tRNAs exhibit different degrees of sensitivity to changes in cation concentration. Peak A, the farthest downfield resonance in the spectra of each fluorinated tRNA, is the most sensitive to changes in monovalent and divalent cation concentration (Figs. 9, 10 and 12). This appears to be a characteristic of all FUra-substituted tRNAs, and may indicate that peak A corresponds to an invariant base substituted by fluorouracil. Redfield and collaborators (Johnson and Redfield, 1981; Tropp and Redfield, 1981) have observed a resonance in the $^1$H NMR spectra of several tRNAs that also exhibits a hypersensitivity to changes in Mg$^{++}$. This peak has been
assigned to the imino proton at N(1) of ψ55. The fluorine atom in FUra 55 is located at the same position normally occupied by the N(1)-H of ψ55. These observations suggest that peak A in the $^{19}$F spectra of all three purified fluorinated tRNAs can be assigned to FUra 55, which replaces the invariant pseudouridine in the T loop of all tRNAs. This assignment agrees with the previous assignment of peak A in the $^{19}$F spectrum of (FUra)$_t$tRNA$^\text{Val}_t$ to FUra 55, based on the $^{19}$F-$^{19}$F homonuclear NOE observed between peaks A and B in the $^{19}$F NMR spectrum of (FUra)$_t$tRNA$^\text{Val}_t$ (Hardin, 1984).

There is one additional resonance, in the central portion of the $^{19}$F spectra of all three purified (FUra)$_t$tRNAs, that is sensitive to changes in [NaCl] (Figs. 9 and 10). These peaks are labeled F, I, and I in the spectra of FUra-substituted tRNA$^\text{Val}_t$, tRNA$^\text{Met}_f$, and tRNA$^\text{Met}_m$, respectively. Two of these resonances, those in (FUra)$_t$tRNA$^\text{Val}_t$ and (FUra)$_t$tRNA$^\text{Met}_f$, have been assigned to FUra residues in the anticodon loops of these tRNAs based on oligonucleotide binding studies (Figs. 21 and 30). This suggests that the conformation of the anticodon loop may be affected by salt concentration. Addition of an oligonucleotide codon to (FUra)$_t$tRNA$^\text{Met}_f$ does not affect the $^{19}$F NMR spectrum of the initiator tRNA (Fig. 31) as codon binding does with the fluorinated elongator tRNAs (Figs. 18 and 30). There is, however, one resonance in the 4.0-5.0 ppm range of the $^{19}$F spectrum of (FUra)$_t$tRNA$^\text{Met}_f$, peak I, that shifts upfield with increasing [NaCl]. Because the chemical shift position of this resonance is similar to the signals assigned to the anticodon of (FUra)$_t$tRNA$^\text{Met}_m$ and (FUra)$_t$tRNA$^\text{Val}_t$, and since all of these peaks display similar behavior with respect to changing [NaCl], it is likely that peak I in the $^{19}$F NMR spectrum of (FUra)$_t$tRNA$^\text{Met}_f$ also represents the FUra residue in the anticodon of the initiator tRNA, FUra 36.

Examination of the magnesium dependence of the chemical shift posi-
tions of the $^{19}$F resonances in the spectrum of (FUra)\textsubscript{tRNA$^{Val}$} (Fig. 12B) has aided in determining the location of all the signals in the spectrum obtained at high magnesium concentration. This is especially true for peaks K and F, which are not resolved in the high magnesium spectrum but are in the absence of Mg$^{++}$. The spectra recorded between 30 and 50 Mg$^{++}$/tRNA clearly show that, at high levels of Mg$^{++}$, there are three $^{19}$F resonances in the cluster of peaks between 4.3 and 4.7 ppm (labeled D, E/F), however, the exact location of peak F at the highest concentration of magnesium is difficult to specify.

A fractional peak is often seen at 4.85 ppm in the $^{19}$F spectrum of FUra-substituted valine tRNA (visible in several of the spectra presented in this study). This small peak is visible at all levels of magnesium and is not affected by changes in divalent cation concentration (Fig. 12A). The significance of this peak is not clear at present. It has been suggested that it may represent a small percentage of a denatured form of the tRNA, because the chemical shift position of this $^{19}$F peak is nearly identical to that of heat denatured (FUra)\textsubscript{tRNA$^{Val}$} (C. C. Hardin, Dept. of Chemistry, Univ. of California, personal communication).

Examination of the effects of paramagnetic metal-ion binding to tRNA can aid in assigning resonances in the NMR spectra (Hurd et al., 1979; Schweizer, 1980). Resonances from fluorines close to the ion binding site are affected most by the bound paramagnetic ion. Crystallographic studies have shown that Mn$^{++}$ binds to the hinge region of tRNA$^{Phe}$ where the D and T loops interact (Fig. 1B) (Jack et al., 1977). The manganese is bonded directly to the N7 of G20, and water ligands of the manganese ion form a number of hydrogen bonds with nearby residues (Jack et al., 1977). $^1$H NMR studies indicate that, in solution, the initial binding of manganese is near the s$^4$U8-A14 and G15-C48 tertiary hydrogen bonds, and based on this observation is was suggested that Mn$^{++}$ binds on the other side of the
hinge region, near phosphates 8 and 9 (see Fig. 1B) (Hurd et al., 1979).

13C NMR studies of [4-13C] uracil-enriched trNAVal have also shown that resonances from uracils in and around the hinge region of the tRNA are affected by Mn** binding (Schweizer, 1980).

Peaks J, L, and N, in the upfield portion of the 19F spectrum of (FUra)trNAVal, are the most sensitive to Mn** binding. This indicates that these resonances may be from FUra residues located near the hinge region of trNAVal, which includes residues 8, 12, 47, and possibly 7, and 59 (FUra 17 is also in this region of the molecule but has already been assigned to peak D, which is not affected by Mn**). Peak J is also affected by Mg** (Fig. 12) but not by monovalent Na** (Fig. 10) or the polycation spermine (Fig. 14). This resonance is among those most affected by photoreaction with HMT (Fig. 17) indicating FUra J is located in a helical region of the tRNA. Peak N and especially peak L are specifically affected by ethidium bromide (Chu and Horowitz unpublished observations), which suggests that these FUra residues are located in the acceptor stem based on comparison to results of 1H NMR studies (B. Reid personal communication); possibly FUra 7 and 67, which are the closest FUra residues in the acceptor stem to the hinge region.

The two other 19F resonances that are paramagnetically broadened by Mn** are peaks A and B. These resonances have been tentatively assigned to FUra 55 and 54 respectively, in the T loop of the molecule. Interestingly one of these two 19F signals, peak A, is hypersensitive to changes in cation concentration (Figs. 12 and 10), whereas the other, peak B, is very insensitive to changes in the concentration of cations (Figs. 12 and 10). 13C NMR studies also detect paramagnetic broadening of signals from residues 54 and 55 in [4-13C] uracil-labeled E. coli trNAVal. (Schweizer, 1980).

Two bound spermine molecules are found in the crystal structure of
tRNA^Phe (Fig. 1B), one bound to the anticodon stem, and the other is found near the variable loop. Hyde and Reid (1985) found specific effects of spermine on the \(^1\text{H}\) NMR spectrum of \(E. \text{coli}\) tRNA^Phe occurring at base-pairs G15-C48 and s^U8-A14. Addition of spermine to (FUr\text{a})tRNA^Val affects three resonances in the \(^{19}\text{F}\) spectrum (Fig. 14). Peak A, (tentatively assigned to FUr\text{a} 55) shifts upfield with increasing spermine. This appears to be a nonspecific effect since this resonance shows similar behavior upon increasing [NaCl] (Fig. 10) or upon removal of Mg\(^{++}\) (Fig. 12). The other two spermine sensitive resonances are peaks G and I. One of these resonances may represent FUr\text{a} 8, which is the closest FUr\text{a} base to the region affected by spermine in the proton NMR studies (Hyde and Reid, 1985). However, further studies are clearly required to assign a \(^{19}\text{F}\) signal to position 8 in (FUr\text{a})tRNA^Val. It is interesting to note that peak G is the one \(^{19}\text{F}\) signal in the central portion of the spectrum that is not sensitive to changes in pH (Fig. 8) or reactive with bisulfite (Fig. 16).

**Codon Anticodon Interaction in FUr\text{a}-Substituted tRNAs**

Because the accuracy of translation is dependent on proper recognition of a codon by its complementary anticodon, the codon-anticodon interaction is one of the most important steps in protein synthesis. In this study, \(^{19}\text{F}\) NMR was used to investigate the interaction of FUr\text{a}-substituted transfer RNA with oligonucleotide codons. The results permit assignment of several \(^{19}\text{F}\) resonances in the spectra to a FUr\text{a} residue in the anticodon of fluorinated tRNA^Val and tRNA^Met, and the determination of binding constants for the interaction between these tRNAs and oligonucleotide codons. The results also provide information about the structure of the anticodon loop.

Addition of codon GpU\text{p}A to (FUr\text{a})tRNA^Val results in the upfield shift
of a single $^{19}\text{F}$ resonance at 3.9 ppm (Fig. 18). Measurement of the shift as a function of $\text{G}_p\text{UpA}$ concentration allows determination of an association constant of $2500 \text{ M}^{-1}$ (Fig. 19), which is just slightly lower than a previously reported value of $3000 \text{ M}^{-1}$ for the association of $\text{G}_p\text{UpA}$ with normal tRNA$^\text{Val}$ (Pongs and Griese, 1972). The difference could be due to the lower temperature and higher salt concentration used by Pongs and Griese (1972), as both conditions are known to increase the affinity constants of these interactions (Uhlenbeck, 1972). Normal tRNA$^\text{Val}$ has a modified nucleoside, uridine-5-oxyacetic acid, at position 34 in the anticodon, whereas in (FUra)tRNA$^\text{Val}$ this is replaced by 5-fluorouridine (Horowitz et al., 1974). This substitution does not appear to significantly affect the affinity of codon.

The sequence complementary to $\text{G}_p\text{UpA}$ occurs only once in tRNA$^\text{Val}$, at the anticodon. This fact, together with the observation that trinucleotides not complementary to the anticodon do not shift the $^{19}\text{F}$ resonance at 3.90 ppm, and the close agreement of the binding constant determined in these studies with the value in the literature, determined by equilibrium dialysis, indicates that the observed effect is due to the binding of $\text{G}_p\text{UpA}$ to the anticodon of (FUra)tRNA$^\text{Val}$. Further evidence to support this conclusion comes from the observation that formation of an anticodon-anticodon complex (Eisinger, 1971; Grosjean et al., 1976) between (FUra)tRNA$^\text{Val}$ (anticodon FAC) and E. coli tRNA$^\text{Lys}$ (anticodon QUA) produces a similar upfield shift of peak H (Fig. 24). On the basis of these results, the resonance at 3.90 ppm can be assigned to FUra-34 in the anticodon loop of (FUra)tRNA$^\text{Val}$. Such an assignment is consistent with evidence, discussed previously, which indicates that the peaks in the central region of the $^{19}\text{F}$ NMR spectrum, including peak H, are derived from FUra residues in single-stranded regions of the tRNA. FUra H is accessible to pH titration, having a pKa of 7.6, similar to that of free 5-
fluorouridine (Fig. 8). Determination of the degree of solvent exposure by measurement of the solvent isotope shift (SIS) on transfer of the tRNA from H_2O to 2H_2O (Hardin, 1984), indicates that FUra H has a SIS value close to that of free 5-fluorouridine and is, by this criterion, completely exposed. Furthermore, this FUra residue readily forms an adduct with bisulfite (Fig. 16), which preferentially reacts with pyrimidines in single-stranded regions of tRNA (Furuichi et al., 1970).

Several studies, including psoralen photoaddition (Fig. 17) and ethidium bromide intercalation (W. C. Chu and J. Horowitz, Dept. of Biochemistry and Biophysics, Iowa State Univ., unpublished results) indicate that the resonances in the upfield portion (above 3.5 ppm) of the 19F spectrum of (FUra)tRNA_{Val} are derived from FUra residues located in helical regions of the tRNA. It follows that when the environment of a FUra residue is changed from single-stranded to base-paired, an upfield shift occurs, as when GpUpA is bound to the anticodon.

Binding of G_pU_pA_pA to (FUra)tRNA_{Val} also shifts the 19F peak at 3.90 ppm upfield (Fig. 21), which indicates that this tetranucleotide also binds to the anticodon region of the tRNA. Additional evidence to support this conclusion comes from the observation that G_pU_pA_pA binding affects the 1H NMR signal from m^6A-36, 3'-adjacent to the anticodon in tRNA_{Val}. It was also shown that ribonuclease H cleaves the complex of dG_pT_pA_pA:(FUra)tRNA_{Val} only at position 34 in the anticodon (Fig. 28), and since dG_pT_pA_pA has similar effects on the 19F NMR spectrum as G_pU_pA_pA, it is reasonable to conclude that the codon-containing oligoribonucleotides also bind only at the anticodon of (FUra)tRNA_{Val}.

The tetranucleotide G_pU_pA_pA binds to the anticodon of (FUra)tRNA_{Val} much more tightly than the codon triplet G_pU_pA does. This increased binding affinity may be due to formation of a fourth base-pair, since the 3'-terminal adenosine of G_pU_pA_pA is complementary to the FUra residue at
position 33, adjacent to the 5' end of the anticodon. However, an alternative explanation for the increased binding is that the 3'-terminal adenosine stabilizes the binding by stacking on the preceding pair (3'-dangling end effect), rather than by forming a base-pair.

In addition to the upfield shift of peak H, G\textsubscript{p}U\textsubscript{p}A\textsubscript{p} binding induces a downfield shift of a second resonance at 4.5 ppm (peak F) (Fig. 21). It is possible that this shift reflects base-pairing between FUra-33 and the 3'-terminal adenosine of the tetranucleotide. If this is true, then peak F can be assigned to FUra-33 of (FUra)\textsubscript{tRNA}\textsuperscript{Val}, and this would also indicate that in solution the anticodon loop can undergo a conformational change from the 3'-stacked conformation observed in the crystal structure (Fig. 1B).

Because G\textsubscript{p}U\textsubscript{p}A\textsubscript{p}A binds to the anticodon with over twice the affinity of G\textsubscript{p}U\textsubscript{p}A, it is possible that the downfield shift of peak F is caused only by the tighter binding. However, formation of an anticodon-anticodon complex between (FUra)\textsubscript{tRNA}\textsuperscript{Val} and \textit{E. coli} tRNA\textsuperscript{Iyr} shifts peak H upfield but does not affect peak F (Fig. 24). Association constants for formation of such complexes are several orders of magnitude greater than those for complementary oligonucleotide binding to the anticodon (Grosjean et al., 1976). This shows that the downfield shift of peak F cannot be attributed solely to the affinity of G\textsubscript{p}U\textsubscript{p}A\textsubscript{p}A binding.

Studies of tetranucleotides containing the codon G\textsubscript{p}U\textsubscript{p}A and a 3'-nucleotide of U, G, or C, to (FUra)\textsubscript{tRNA}\textsuperscript{Val} (Figs. 22 and 23; C not shown) show that the effect on the 19F resonance at 4.60 ppm is specific for G\textsubscript{p}U\textsubscript{p}A\textsubscript{p}A, indicating that this shift is not due to a nonspecific interaction of a 3'-dangling nucleoside. On the basis of these results, a preliminary assignment of one of the resonances at ca. 4.60 ppm (peak F) to FUra-33 of (FUra)\textsubscript{tRNA}\textsuperscript{Val} can be made.

Addition of 3'-dangling ends to the codon does increase the binding
affinity of all the resulting tetranucleotides as compared to GpuA. This is especially evident in the case of GpuApG, which binds with higher affinity than either GpuApC or GpuApU, and, with an association constant twice that of GpuA, GpuApG binds nearly as tightly as GpuApA. These findings agree with previous studies that have shown that purines are more efficient than pyrimidines at stabilizing short RNA duplexes as 3'-dangling ends (Westhof et al., 1983; Freier et al., 1983, 1985).

Support for the assignments of peak H and peak F to the FUra residues in the anticodon of (FUra)trNA\textsubscript{val} comes from the observation that both of these peaks are specifically affected when the tRNA is hydrolyzed by S1 nuclease under conditions which lead to cleavage in the anticodon loop (P. Gollnick and J. Horowitz, Dept. of Biochemistry and Biophysics, Iowa State Univ., unpublished observations). In spectra recorded with or without Mg\textsuperscript{++}, both peaks show a loss of intensity as compared to untreated (FUra)trNA\textsubscript{val} presumably due to partial removal of FUra-33 and FUra-34 by the nuclease. Peak F is also shifted downfield ca. 0.3 ppm in the spectrum of nuclease S1 cleaved tRNA recorded in 15 mM MgCl\textsubscript{2}, possibly reflecting an altered environment resulting from cleavage of the loop.

Formation of a base-pair between FUra-33 and the tetranucleotide requires that the anticodon loop undergo a conformational change from the 3'-stacked conformation observed in the crystal structure (Fig. 1B), perhaps to a 5'-stacked conformation first proposed by Fuller and Hodgson (1967) (see Fig. 33). As discussed in the introduction, there is experimental evidence for conformational flexibility in the anticodon loop. Several NMR studies have provided evidence for multiple conformations in this loop (Gorenstein and Goldfield, 1982; Salemink et al., 1980). The temperature dependence of fluorescence from the wye base in the anticodon loop of yeast trNA\textsuperscript{Phe} shows structural transitions characterized as an "all-or-none" effect, which have been suggested to reflect a transition
Figure 33. Possible model for the conformational change in the anticodon loop
from the 3'-stacked to the 5'-stacked anticodon (Urbake and Maass, 1978). Several oligonucleotide binding studies also suggest that the anticodon can exist stacked on the 5'-side of the loop (Uhlenbeck et al., 1970; Eisinger and Spahr, 1973; Yoon et al., 1975; Geerdes et al., 1980a). The most convincing of these studies used $^1$H NMR to demonstrate that four to five base-pairs are formed between the codon-containing pentanucleotide $U_pU_pC_pA_pG$ and the anticodon loop of $tRNA^{Phe}$ Geerdes et al., 1980a).

The two resonances in the $^{19}$F NMR spectrum of $(F\text{Ura})tRNA^{Val}$ that have been assigned to the anticodon loop, peaks F and H, are the most sensitive to changes in temperature, either in the presence (Fig. 15) or absence (Hardin, 1984) of magnesium. This behavior also suggests conformational flexibility in the anticodon loop.

The exact nature of the interaction between the 3'-terminal adenosine of $G_pU_pA_pA$ and $F\text{Ura-33}$ is somewhat unclear. For one thing, the shift of peak F upon binding the tetranucleotide is downfield (Fig. 21), rather than upfield as seen with peak H upon codon binding (Figs. 18 and 21). The downfield shift of peak F may reflect a conformational change in the anticodon loop near $F\text{Ura-33}$, whereas the upfield shift of peak H only reflects the change due to base-pairing with the oligonucleotide. It is also possible that the shift of peak F is due to a conformational change in other parts of the molecule. While this cannot be completely ruled out, it seems unlikely since binding of $G_pU_pA$ or $G_pU_pA_pX$ ($X=U, G, \text{or } C$) or formation of an anticodon-anticodon complex with $tRNA^{Tyr}$ does not affect peak F.

Addition of $U_pA_pA$, which is a constituent of $G_pU_pA_pA$, and is complementary to $F\text{Ura-33}$ and the first two residues of the anticodon, does not perturb the $^{19}$F spectrum of $(F\text{Ura})tRNA^{Val}$ (Fig. 20). This may be due to insufficient binding affinity, because of the lack of a G-C base pair, or $U_pA_pA$ may not bind due to the conformation of the anticodon loop, i.e., if
the loop exists in the 3' stack, position 33 is not available for binding (see Fig. 33). If UpA_pA doesn't bind to (FUr)RNAVal due to the conformation of the loop, then this suggests that G_pUpA_pA binding induces a conformational change which allows the 3'-terminal adenosine to base-pair with FUra-33.

The conformation of the anticodon loop in the crystal structure of tRNA^Phe (Fig. 1B) is stabilized by a magnesium hydrate ion; Mg^{++} binds to a phosphate oxygen of residue 37, and the water molecules, coordinated to the Mg^{++} ion, form several hydrogen bonds to nearby bases of the loop (Holbrook et al., 1977; Quigley et al., 1978). It has been suggested that removal of the magnesium ion would result in a more flexible anticodon loop (Kim, 1980; Teeter et al., 1980).

The results of codon binding to (FUr)RNAVal in the absence of magnesium are consistent with an alternate anticodon loop structure. First, the binding constants obtained for either the tri- or tetranucleotide codon are much lower than those obtained in 15 mM Mg^{++}. This may be a result of a change from the conformation of the anticodon loop seen in the crystal structures (Fig. 1B), in which the anticodon is stacked perfectly to interact with a codon, to a structure less suited for codon binding. Also, G_pUpA binding in the absence of Mg^{++} shifts both peaks F and H, whereas in the presence of magnesium only peak H is affected. If the structure of the anticodon loop in the presence of magnesium resembles that seen in the crystal (Fig. 1B) binding of a codon trinucleotide might affect the environment of the base at position 34 while position 33 remains unperturbed. This is because residue 33 is separated from the anticodon by a sharp bend in the polynucleotide backbone. Removal of magnesium may alter the conformation of the anticodon loop in such a way so that U-33 is no longer separated from the anticodon, and codon binding now perturbs the environment of this base.
GpUpApA binding also affects peak F differently in the absence of magnesium, causing an upfield shift rather than the downfield shift observed in 15 mM Mg\(^{2+}\). Earlier it was suggested that the upfield shift of peak H upon codon binding reflects a change in the environment of FUra-34 from single-stranded to hydrogen-bonded, and that the downfield shift of peak F may be due to a more complicated change in the structure around FUra-33, possibly involving a conformational change from the 3' stack. The upfield shift of peak F upon addition of GpUpApA in the absence of Mg\(^{2+}\) clearly indicates that a different type of interaction occurs between the tetranucleotide and the anticodon in the absence of Mg\(^{2+}\) than in the presence of magnesium. The upfield shift of peak F may indicate that at low Mg\(^{2+}\) the anticodon loop structure is such that the 3'-terminal adenosine of GpUpApA can base-pair directly with FUra-33 without any conformational change.

In the crystal structure of yeast tRNA\(^{Phe}\), the conformation of the anticodon arm is affected by the presence of a spermine molecule bound to the anticodon stem (Fig. 1B). The positively charged polycation draws the anionic phosphate groups on the opposite sides of the major groove together, and produces a bend in the double helix. Together with the bound magnesium, spermine has the effect of substantially immobilizing the anticodon (Teeter et al., 1980). Fluorescence studies of spermine binding to a tRNA\(^{Phe}\) derivative carrying ethidium at position 37 in the anticodon have shown that spermine stabilizes one particular conformation of the anticodon loop (Nilsson et al., 1983). These studies also show that the polyamine stabilizes the same structure as Mg\(^{2+}\) does, but the mechanism of action is different.

Addition of GpUpApA to FUra-substituted tRNA\(^{Val}\) in the presence of spermine results in only the upfield shift of peak H, assigned to FUra-34 (Fig. 29). Peak F, at 4.60 ppm, was not affected by GpUpApA in the
presence of spermine, as it was in the absence of the polyamine (compare Fig. 29 to Fig. 21). If, as has been suggested, the downfield shift of peak F reflects pairing of 

$\text{FUra-33 with GpUpApA}$, then the lack of any effect in the presence of spermine suggests that spermine stabilizes a conformation of the anticodon in which 

$\text{FUra-33 is unable to interact with an oligonucleotide bound to the anticodon, possibly the 3'-stacked conformation seen in crystals grown in the presence of spermine (Fig. 1B).}$

Binding of the trinucleotide codon $\text{A}_p\text{U}_p\text{G}$ to $(\text{FUra})\text{tRNA}_{m}^\text{Met}$ also induces an upfield shift of one resonance, peak I, in the central (4.0-5.5 ppm) region of the $^{19}\text{F NMR}$ spectrum of this tRNA (Fig. 30). Because the shift is codon-specific, and the calculated binding constant agrees well with that in the literature, a preliminary assignment of peak I to Fura-36 at the 3' position of the anticodon of $(\text{FUra})\text{tRNA}_{m}^\text{Met}$ can be made.

Interestingly, no changes in the $^{19}\text{F NMR}$ spectrum of FUra-substituted $\text{E. coli}$ initiator tRNA$_{f}^{\text{Met}}$ are observed upon addition of the codon $\text{A}_p\text{U}_p\text{G}$ (Fig. 31) or $\text{A}_p\text{U}_p\text{GpA}$ (data not shown). This is surprising in view of an earlier report which showed that the affinity of $\text{E. coli}$ tRNA$_{f}^{\text{Met}}$ for $\text{A}_p\text{U}_p\text{G}$ is three times greater than that of tRNA$_{m}^\text{Met}$ (Hogenauer et al., 1972). The anticodon loop of the $(\text{FUra})\text{tRNA}_{f}^{\text{Met}}$ used in these experiment was intact, as judged by polyacrylamide gel electrophoresis under denaturing conditions (7 M urea) (Data not shown). This would indicate two possible explanations for the lack of any effect of $\text{A}_p\text{U}_p\text{G}$ on the spectrum of $(\text{FUra})\text{tRNA}_{f}^{\text{Met}}$: a low binding constant for codon; or, in contrast to the results with tRNA$_{f}^{\text{Val}}$ and tRNA$_{m}^{\text{Met}}$, binding of the oligonucleotide to tRNA$_{f}^{\text{Met}}$ does not affect the magnetic environment of FUra-36 in the anticodon. Subtle conformational differences between the anticodon loop of initiator tRNAs and elongator tRNAs have been detected by X-ray diffraction analysis of tRNA crystals (Woo et al., 1980) and by enzymatic (nuclease S1) analysis (Wrede et al., 1979). It is also possible that
incorporation of 5-fluorouracil has affected the structure of the anti-
codon loop resulting in a conformation unfavorable for codon binding.
Other studies have indicated that (FUra)\(_{\text{tRNA}^\text{Met}}\) interacts with initiation
factor in an unusual manner (J. Horowitz, Dept. of Biochemistry and
Biophysics, Iowa State Univ., and I. Schwarz, New York Med. Col.,
unpublished results).

\(^{19}\text{F} \text{chemical shifts are extremely sensitive to the environment of the}
fluorine atom. Yet oligonucleotide codon binding to (FUra)\(_{\text{tRNA}^\text{Val}}\) and
(FUra)\(_{\text{tRNA}^\text{Met}}\) results in the shift of only one or two peaks in the \(^{19}\text{F} \text{NMR}
spectra of these tRNAs, presumably the FUra residues located in the anti-
codon. No other significant changes in the spectra are observed. There
is no evidence for trinucleotide codon-induced aggregation of (FUra)\(_{\text{tRNA}}\) such as that observed with several other tRNAs (Porschke and Labuda, 1982;
Geerdes et al., 1980b; Clore et al., 1984); the resonances in the \(^{19}\text{F} \text{NMR}
spectra are not broadened by codon binding (Figs. 18, 21, and 30), as they
are upon formation of an anticodon-anticodon dimer between (FUra)\(_{\text{tRNA}^\text{Val}}\) and
\(_{\text{tRNA}^\text{Try}}\) (Fig. 20).

There are no indications of more far reaching conformational changes
in the tRNA such as a disruption of the interactions between the D and T
loops. This conclusion is supported by the lack of any effect of CpGpApA
addition on the \(^{19}\text{F} \text{NMR spectrum of (FUra)\(_{\text{tRNA}^\text{Val}}\)}, either in the absence
or presence of the codon GpUpA (Fig. 32), and similar results were also
obtained using a pentanucleotide codon GpUpApApA (results not shown). A
number of experiments, discussed previously, have suggested that the two
farthest downfield peaks in the \(^{19}\text{F} \text{spectra of (FUra)\(_{\text{tRNA}^\text{Val}}\) represent}
FUra 54 and 55 (Hartin, 1984). Codon binding, either oligonucleotide
codons, or the complementary anticodon of \textit{E. coli} \(_{\text{tRNA}^\text{Tyr}}\), has no effect
on peak A or B in the \(^{19}\text{F} \text{NMR spectrum of (FUra)\(_{\text{tRNA}^\text{Val}}\)} \text{ (Figs. 18, 21 and
24). Binding of the tetranucleotide to the FpFpCpG in the T loop should}
certainly have resulted in the shift of one or more $^{19}$F signals, as occurs when oligonucleotide codons bind to the anticodon of several FUra-substituted tRNAs. The absence of such shifts indicates that the $F_pF_pC_pG$ sequence is not available for oligonucleotide binding, even when codon is bound to the anticodon. This is consistent with the results of proton NMR studies (Geerdes et al., 1978, 1980a; Davanloo et al., 1979). The possibility of small conformational changes, which do not change the magnetic environment of any of the incorporated fluorines in the tRNA cannot be ruled out.

A high concentration of $C_pG_pA_pA$ does, however, cause the shift of several resonances in the 2.5-3.5 ppm region of the $^{19}$F spectrum of (FUra)tRNA$^\text{Val}_1$ when the anticodon of the tRNA is bound in a tight complex to the complementary anticodon of E. coli tRNA$^\text{Tyr}_2$ (data not shown). The resonances affected, however, are not those corresponding to the FUra residues at positions 54 and 55 in the T loop (peaks A and B). Further investigation will be necessary to determine the nature of the structural change and whether this represents a specific effect of $C_pG_pA_pA$. 

REFERENCES


ACKNOWLEDGMENTS

This is dedicated to my parents, Phil and Doris, in thanks for all their support and encouragement. Also, I thank my brother Chuck for his help with computer related problems, and especially for his program to handle references.

To Dr. Horowitz, my sincere appreciation for all his guidance and his patience throughout this work.

Special thanks to my wife Sandy for all her support and patience. I would also like to thank her parents, Al and Sally, my "Iowa family".

I would like to thank Jennifer Morris and Michelle Smart for their excellent technical assistance, especially for preparing the fluorouracil-substituted tRNA\textsuperscript{Val} used in these studies. Also I thank Dave Scott for his help with the NMR spectrometer.

Thanks to all the 306 gang, especially Tom Girard, who kept me sane, even when things didn't go so well.

Finally, I would like to thank two very special teachers who influenced my decision to study biochemistry. First, George Johnson, my outstanding high school chemistry teacher; and second Dr. Bruce McFadden, who gave me my first chance to do research, and whose continued support is deeply appreciated.