Partial Assignment of Resonances in the ^{19}F Nuclear Magnetic Resonance Spectra of 5-Fluorouracil-Substituted Transfer RNAs†

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ABSTRACT: Features of the ^{19}F nuclear magnetic resonance (NMR) spectra of three purified 5-fluorouracil-(FUra-)substituted *Escherichia coli* tRNAs, $tRNA_1^{Val}$, $tRNA_m^{Met}$, and $tRNA_f^{Met}$, are compared. Each of the tRNA species can be resolved into two isoaccepting forms, A and B, whose ^{19}F NMR spectra differ in the shift of one peak from the 4.5 to 4.8 parts per million (ppm) range (FUra = 0) in the spectrum of isoacceptor B upfield to ca. −15 ppm in that of isoacceptor A. Because the sequences of the two isoacceptors of each tRNA differ only at one position in the D loop, that normally occupied by a dihydrouridine residue, we assign the 4.5 ppm peak in the spectrum of fluorine-labeled $tRNA_1^{Val}$ to FUra17 and the resonance at 4.6 ppm in the spectrum of fluorouracil-substituted $tRNA_m^{Met}$ to FUra20. A reciprocal $^{19}F\{^{19}F\}$ nuclear Overhauser effect is observed between the downfield peaks A and B in the ^{19}F NMR spectrum of ^{19}F-labeled $tRNA_1^{Val}$. Assuming that fluorine-labeled $tRNA_1^{Val}$ has a structure similar to that of yeast $tRNA^{Phe}$, only FUra54 and -55 are close enough (4–5 Å) to give an appreciable ^{19}F homonuclear Overhauser effect. Peaks A and B have therefore been assigned to FUra54 and -55. As the temperature is raised from 30 to 45 °C, the intensity of peak B (6.6 ppm) in the spectrum of ^{19}F-labeled $tRNA_1^{Val}$ gradually shifts upfield to 6.4 ppm (T_m = 36 °C), indicating a temperature-dependent slow exchange of the corresponding 5-fluorouracil residue between two magnetically distinct environments. Because this effect resembles the splitting of T54 methyl ^1H and ^{13}C signals in the spectra of several native tRNAs [Kastrup, R. V., & Schmidt, P. G. (1978) *Nucleic Acids Res. 5*, 257–269; Kopper, R. A., Schmidt, P. G., & Agris, P. F. (1983) *Biochemistry 22*, 1396–1401], we assign peak B to FUra54. Peak A in the spectrum of ^{19}F-labeled $tRNA_1^{Val}$ can then be assigned to FUra55. The lowest field resonance (peak A) in the ^{19}F spectra of each of the three tRNAs exhibits a uniquely large chemical shift change with changing ionic strength or magnesium ion concentration. This similarity suggests that peak A corresponds to a conserved base in the tRNAs and is consistent with assignment of peak A in the ^{19}F NMR spectra of all three fluorinated tRNAs to the 5-fluorouracil residue that replaces the invariant Ψ55.

Nuclear magnetic resonance (NMR)[1] spectroscopy is a powerful method for examining the structure, conformation, and dynamic properties of tRNA in solution (Reid, 1981). ^1H is the magnetic nucleus most frequently observed; however, ^{31}P (Gorenstein, 1984), ^{13}C (Kopper et al., 1983; Olsen et al., 1982), and ^{15}N (Griffey et al., 1983) have also been used. Each of these nuclei has inherent advantages and disadvantages [see Hardin et al. (1986)]. To circumvent some of the difficulties, we have made use of ^{19}F NMR to probe the structure of tRNAs labeled in vivo with 5-fluorouracil (Horowitz et al., 1977; Hills et al., 1983). A number of recent reports demonstrate that significant structural and dynamic information can be obtained by this approach (Gollnick et al., 1986, 1987; Hardin et al., 1987; Hardin & Horowitz, 1987). Advantages of using a fluorine-19 probe include 100% natural abundance, a high signal sensitivity (83% that of ^1H), and a large chemical shift range compared with protons (Sykes & Hull, 1978; Gerig, 1978; Sykes & Weiner, 1980). It has also been shown that chemical shifts and relaxation rates are more sensitive to the chemical environment for ^{19}F than for ^1H or ^{13}C because of the importance of the chemical shift anisotropy relaxation mechanism (Hull & Sykes, 1975; Hardin & Horowitz, 1987). Furthermore, because fluorine is not normally found in proteins, ^{19}F NMR spectroscopy is especially well suited to monitor the specific interaction of fluorinated tRNAs with proteins such as aminoacyl-tRNA synthetases and elongation factor EF-Tu. In a recent ^{19}F NMR study of λ phage *cro* repressor interaction with synthetic 2′-deoxy-5-fluorouracil-containing O_R3 operator DNA, Metzler et al. (1985) demonstrated the exceptional sensitivity of ^{19}F chemical shifts to protein binding.

5-Fluorouracil-substituted tRNAs are well suited to investigation by ^{19}F NMR because they are highly labeled, having 90–95% of their uracil and uracil-derived minor bases replaced by FUra (Lowrie & Bergquist, 1968; Johnson et al., 1969; Kaiser, 1972). The covalently bound fluorine atom is nearly isosteric with a hydrogen atom and is a minimally perturbing probe of nucleic acid structure. Physical properties of ^{19}F tRNAs are similar to those of their normal counterparts (Horowitz et al., 1974), and the incorporation of FUra into

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[1] Abbreviations: FUra, 5-fluorouracil; (FUra)$tRNA_1^{Val}$, 5-fluorouracil-substituted *E. coli* $tRNA_1^{Val}$; (FUra)$tRNA_f^{Met}$, 5-fluorouracil-substituted *E. coli* $tRNA_f^{Met}$; (FUra)$tRNA_m^{Met}$, 5-fluorouracil-substituted *E. coli* $tRNA_m^{Met}$; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; NMR, nuclear magnetic resonance; ppm, parts per million; NOE, nuclear Overhauser effect; T, ribothymidine; Ψ, pseudouridine; s^4U, 4-thiouridine.

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synthetic DNA oligonucleotides has little effect on their observed temperature-dependent dynamics and NMR melting temperatures (Kremer et al., 1987). Moreover, with a few exceptions (Ramberg et al., 1978), fluorinated tRNAs retain full activity in protein synthesis and other tRNA-dependent biochemical functions (Horowitz et al., 1974; Ofengand et al., 1974; Chinalli et al., 1978). The incorporated ^{19}F label is located in loop, stem, and tertiary structural regions of the tRNA. ^{19}F NMR thus provides a unique way to obtain conformational and dynamic information on bases in loop or tertiary structures that cannot be readily monitored by ^{1}H NMR (Gollnick et al., 1986, 1987). The ^{19}F NMR spectra of two FUra-substituted *Escherichia coli* tRNAs have been described, $tRNA_1^{Val}$ (Horowitz et al., 1977; Hardin et al., 1986) and $tRNA_f^{Met}$ (Hills et al., 1983). Each tRNA gives a characteristic ^{19}F spectrum, spread over a 7.5 ppm range, showing resolved resonances for virtually all incorporated fluorouracils.

Detailed studies with (FUra)$tRNA_1^{Val}$ (Hardin et al., 1986; Hardin & Horowitz, 1987; Gollnick et al., 1986, 1987) demonstrated that the ^{19}F chemical shifts and relaxation parameters are very sensitive to changes in tRNA structure. To interpret these changes at the moleclar level, it is essential to have realiably assigned ^{19}F resonances. This paper presents evidence for a number of specific peak assignments. Two isoaccepting forms of a third FUra-substituted *E. coli* tRNA, $tRNA_m^{Met}$, as well as a second isoacceptor of the previously described ^{19}F-labeled $tRNA_1^{Val}$, have been isolated. By comparing the ^{19}F NMR spectra of these tRNAs as a function of ionic strength and divalent cation concentration, and analyzing the results of chemical cleavage experiments, thermal denaturation studies, and ^{19}F homonuclear NOE measurements, we are able to assign several resonances in the ^{19}F spectra.

Experimental Procedures

Materials

RPC-5 adsorbant was prepared according to method C of Pearson et al. (1971); Adogen 464 (Ashland Chemical Co., Columbus, OH) and Plaskon CTFE 2300 powder (Allied Chemical Co., Elizabeth, NJ) were the generous gifts of Dr. Ivan Kaiser, Department of Biochemistry, University of Wyoming, Laramie. Adenosine 5′-[γ-^{32}P]triphosphate was purchased from New England Nuclear. 5-Fluorouracil was donated by Hoffmann-La Roche. Calf intestinal phosphatase (molecular biology grade) was from Boehringer Mannheim, and polynucleotide kinase was a Pharmacia product. Eastman Kodak X-Omat XAR-5 film was used for fluorography and autoradiography. All other enzymes and reagents have been described previously (Hills et al., 1983; Hardin et al., 1986). 6-Deuterio-5-fluorouracil was synthesized by reaction of 5-fluorouracil with NaOD–D_2O (Aldrich) as described by Cushley et al. (1968). The reaction was followed by the disappearance of the H-6 signal in the ^{1}H NMR spectrum and loss of the 7-Hz ^{19}F–^{1}H scalar coupling in the ^{19}F NMR spectrum.

Methods

Preparation of 5-Fluorouracil-Substituted E. coli tRNAs. 5-Fluorouracil-substituted $tRNA_m^{Met}$ was purified from *E. coli* B cells grown in FUra-containing medium (Horowitz et al., 1974) by a series of chromatographic steps involving an initial chromatography on DEAE-cellulose at pH 8.9 to separate normal and FUra-substituted tRNAs. Subsequent purification was achieved by chromatography at 5 °C on a Sepharose 4B column developed with a descending salt gradient from 1.5 to 0 M $(NH_4)_2SO_4$. The pooled $tRNA_m^{Met}$-containing fractions were further purified by elution from DEAE-Sephadex A-50 (at room temperature) by a linear NaCl gradient (0.425–0.5 M) in 20 mM Tris-HCl buffer, pH 7.5, containing 8 mM $MgCl_2$ (Nishimura, 1971). A final purification step involved chromatography at 5 °C on BD-cellulose; the tRNA was eluted with a linear NaCl gradient (0.4–1.0 M) in 10 mM sodium acetate, pH 4.5, containing 10 mM $MgCl_2$. This step separated two isoaccepting forms of the FUra-substituted $tRNA_m^{Met}$ (forms A and B). Only a minimal further purification of either form of the tRNA was achieved by reverse-phase chromatography on RPC-5 (Pearson et al., 1971). The purified A form of $tRNA_m^{Met}$ had a specific activity of 1275 pmol/A_{260} unit, and form B had a specific activity of 1500 pmol/A_{260} unit. Methionine esterified to either isoacceptor of $tRNA_m^{Met}$ could not be N-formylated.

Fluorine-labeled *E. coli* $tRNA_f^{Met}$ was prepared as described by Hills et al. (1983). Two isoacceptors, the A and B forms, were separated on BD-cellulose (Hills et al., 1983). 5-Fluorouracil-substituted *E. coli* $tRNA_1^{Val}$ was purified by the procedure of Hardin et al. (1986). The two isoacceptors of fluorinated $tRNA_1^{Val}$ are not resolved on BD-cellulose but can be separated by chromatography on RPC-5 (Pearson et al., 1971). 6-Deuterio-5-fluorouracil-substituted $tRNA_1^{Val}$ was isolated from *E. coli* cells grown with 6-deuterio-5-fluorouracil, by the same procedure used to prepare the nondeuteriated tRNA. Deuterium was not lost during the chromatography on DEAE-cellulose at pH 8.9, as indicated by the absence of ^{19}F–^{1}H scalar coupling in the ^{19}F NMR spectrum of a RNase P1 hydrolysate of the tRNA after this step.

Amino acid acceptance (Horowitz et al., 1974) of all purified tRNA samples was 1200–1800 pmol/A_{260} unit. Transfer RNA concentrations were determined spectrophotometrically, assuming a value of $E_{260nm}^{0.1\%} = 24$. Nucleoside composition analysis of tRNAs was performed by the ^{3}H derivative method of Randerath et al. (1974).

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in 15% polyacrylamide gels (0.4 × 300 × 310 mm) prepared in electrophoresis buffer consisting of 100 mM Tris–borate, pH 8.3, 2 mM EDTA, and 8 M urea (Maniatis et al., 1982). ^{32}P-Labeled RNA samples were mixed with an equal volume of electrophoresis buffer containing 0.1% bromophenol blue and 0.1% xylene cyanol in 8 M urea, heated at 90 °C for 30 s, and applied to the gel. After electrophoresis at room temperature in the same buffer without urea at 1000–1500 V for 2–6 h, RNA was detected by autoradiography (Maniatis et al., 1982).

Aniline Cleavage of tRNA. The position of the modified fluorodihydrouracil residue in the A form of FUra-substituted tRNAs was determined by aniline cleavage of 5′-^{32}P end-labeled tRNA (Peattie, 1979). Transfer RNA was labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP after dephosphorylation with calf intestinal phosphatase (Silberklang et al., 1979); the labeled tRNA was purified by preparative gel electrophoresis. Samples containing ca. 1 μCi of 5′-^{32}P 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 the mixture was lyophilized to dryness. The residue was redissolved in 20 μL of water, frozen, and lyophilized to dryness twice more to remove all traces of aniline/acetate. The reaction products were separated by electrophoresis on polyacrylamide sequencing gels. Control samples were incubated in 50 mM sodium acetate, pH 4.5, without aniline. A partial RNase T_1 digest of the tRNA, prepared

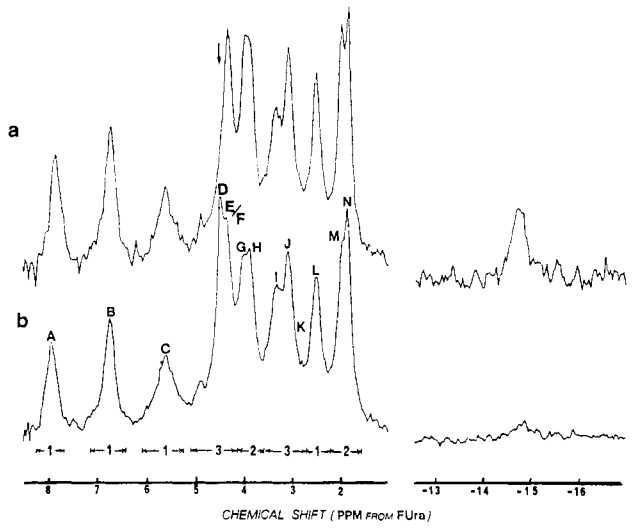

FIGURE 1: ^{19}F NMR spectra of 5-fluorouracil-substituted $tRNA_1^{Val}$. (a) (FUra)$tRNA_1^{Val}$ form A; (b) (FUra)$tRNA_1^{Val}$ form B. The spectra were recorded at 282 MHz in the Fourier-transform mode at 23 °C as described under Methods. Samples were prepared in 50 mM sodium cacodylate, pH 6.0, containing 100 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, and 10% D_2O as an internal lock (standard buffer). Chemical shifts are given in ppm from 5-fluorouracil. Areas under each peak were obtained by integration on the spectrometer and are normalized relative to the area of peak B.

as described by Donis-Keller (1979), provided markers for identifying the site of cleavage.

Temperature–Absorbance Measurements. Absorbance changes of tRNA samples dissolved in degassed 50 mM sodium cacodylate, pH 6.0, 100 mM NaCl, and 1 mM EDTA were monitored at 260 nm with a Gilford Model 250 spectrophotometer. Temperature was controlled by circulating water from a variable-temperature bath through a thermal jacket surrounding the sample chamber. The temperature was increased at a rate of 0.3 °C/min and was monitored via a thermocouple located in the sample chamber.

^{19}F NMR Spectroscopy. Transfer RNA samples (2.5–5.5 mg) were prepared for NMR spectroscopy as described previously (Hardin et al., 1986), and ^{19}F NMR spectra were obtained on a Bruker WM300 pulsed FT NMR spectrometer operating at 282 MHz (Hardin et al., 1986).

^{19}F homonuclear Overhauser effect measurements were made at 282 MHz on 6-deuterio-5-fluorouracil-substituted $tRNA_1^{Val}$ in 100% D_2O, 50 mM sodium cacodylate, pH 6.0, 15 mM $MgCl_2$, 100 mM NaCl, and 1 mM EDTA. The pulse sequence consisted of a 300-ms 45-mW preirradiation pulse centered at the frequency of interest followed by a 4-μs delay, a 90° observation pulse, and a 5-s relaxation delay. Typically, 5000 scans were collected with a sample concentration of approximately 0.7 mM. NOEs were observed by taking the difference between the on-resonance spectrum and a spectrum collected with preirradiation off-resonance, 4 ppm upfield from the FUra standard peak.

RESULTS

Fluorine-19 NMR Spectra of 5-Fluorouracil-Substituted tRNAs. It is generally accepted that all tRNAs have a similar tertiary structure, maintained by hydrogen bonding between conserved and semiconserved bases. Several of these invariant bases are uracil or uracil derivatives, and these are replaced by 5-fluorouracil in FUra-substituted tRNAs. Because of the structural similarities among tRNAs, the peaks corresponding to FUra residues at conserved positions are expected to have similar chemical shifts in the ^{19}F NMR spectra of different

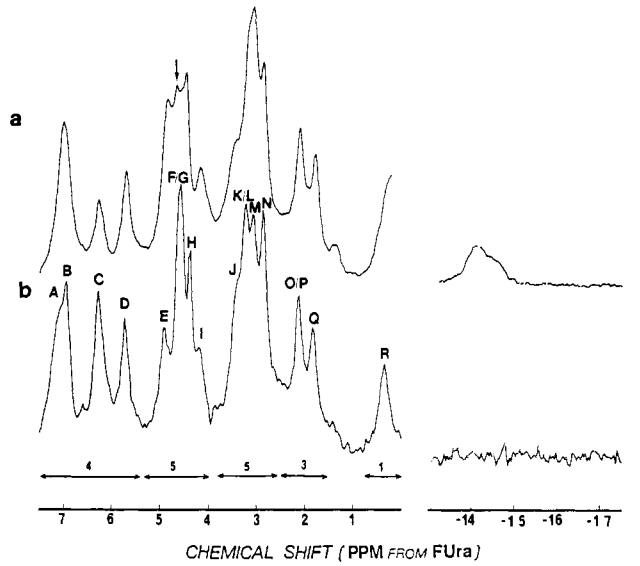

FIGURE 2: ^{19}F NMR spectra of 5-fluorouracil-substituted $tRNA_m^{Met}$. (a) (FUra)$tRNA_m^{Met}$ form A; (b) (FUra)$tRNA_m^{Met}$ form B. Conditions are the same as in Figure 1. Integrals were determined relative to peak D.

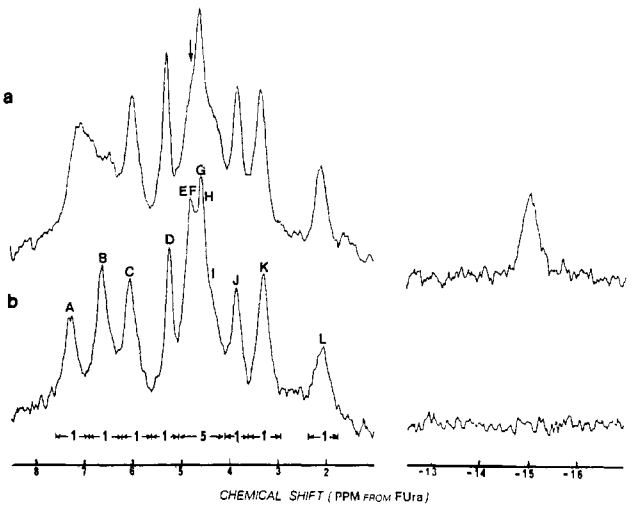

FIGURE 3: ^{19}F NMR spectra of 5-fluorouracil-substituted $tRNA_f^{Met}$. (a) (FUra)$tRNA_f^{Met}$ form A; (b) (FUra)$tRNA_f^{Met}$ form B. Conditions are those described in Figure 1. Integrals were determined relative to peak K.

(FUra)tRNAs. Comparison of the ^{19}F spectra of several fluorinated tRNAs should, therefore, permit identification of these peaks. Three FUra-substituted *E. coli* tRNAs have now been purified: $tRNA_1^{Val}$, $tRNA_f^{Met}$, and $tRNA_m^{Met}$. The preparation and properties of two isoaccepting forms (A and B) of initiator $tRNA_f^{Met}$ were described earlier (Hills et al., 1983). We also previously reported the isolation of (FUra)-$tRNA_1^{Val}$ (Horowitz et al., 1974; Hardin et al., 1986) and have now been able to separate two isoaccepting forms of the tRNA by chromatography on RPC-5 (see Methods). Preparation of the third ^{19}F-labeled tRNA from *E. coli*, the elongator $tRNA_m^{Met}$, is described here for the first time (see Methods). During chromatography on BD-cellulose, (FUra)$tRNA_m^{Met}$, like (FUra)$tRNA_f^{Met}$, is separated into two isoaccepting forms (A and B). Both isoacceptors of this tRNA, like the other purified (FUra)tRNAs (Horowitz et al., 1974; Hills et al., 1983), have more than 95% of their uracil and uracil-derived minor bases replaced by FUra (results not shown; see Methods).

Comparison of the ^{19}F NMR spectra of the two isoacceptors of each tRNA, obtained at room temperature in 15 mM Mg^{2+}

and 100 mM NaCl, is made in Figures 1–3. The spectra of the two forms of (FUra)tRNA$_f^{Met}$ differ somewhat from those previously published (Hills et al., 1983) which were recorded at a lower (10 mM) salt concentration. All spectra were recorded at pH 6.0 to minimize effects due to dissociation of the N(3)-H of incorporated 5-fluorouracil (Hardin et al., 1986).

The ^{19}F NMR spectra of the three tRNAs differ from each other and are characteristic of the individual tRNAs. There are, however, a number of similar spectral features: In the central region, between 4 and 5 ppm, each spectrum has a major cluster of peaks; three or four well-resolved signals are observed downfield from the central cluster (5.5–8 ppm), and a variable number of resonances are found upfield (0.5–4 ppm). The integrals of the peaks in the tRNA spectra, normalized to the integral of an isolated ^{19}F resonance, are also shown. For each tRNA, the total integral agrees with the expected number of FUra residues. There are 12 resolved peaks for the 14 incorporated 5-fluorouracils in fluorinated (FUra)tRNA$_1^{Val}$ (Figure 1). A total of 13 resolved resonances and 2 shoulders (Figure 2) are visible in the ^{19}F spectrum of tRNA$_m^{Met}$ (18 FUra); 9–10 ^{19}F resonances, derived from 12 FUras, are present in the spectrum of (FUra)tRNA$_f^{Met}$ (Figure 3). The spectrum of (FUra)tRNA$_m^{Met}$ exhibits a ^{19}F signal at 0.5 ppm (Figure 2), considerably further upfield than any resonance in the spectra of the other two fluorinated tRNAs. These results demonstrate that it is possible to resolve ^{19}F resonances for virtually all incorporated FUra residues, indicating that each is in a different chemical environment in the folded native structure of the tRNA.

The ^{19}F spectrum of the A form of each tRNA differs from that of the B form primarily in the shift of one resonance from 4.5 to 4.8 ppm (B form), upfield to −15 ppm (A form). This upfield ^{19}F signal is known to be due to a ring-opened fluorodihydrouridine present in isoacceptor A of each tRNA (Horowitz et al., 1983). The ^{19}F peak present in the 4.5–4.8 ppm region of the spectra of the B form of all three tRNAs but absent in the spectra of the A forms (indicated by arrows in Figure 1–3) can thus be attributed to the FUra residue that replaces the fluorodihydrouridine derivative in the corresponding A form.

^{19}F Assignments of FUra in the D Loop. By determining the position of the dihydrofluorouridine residue in each tRNA, we can assign the extra peak in the 4.5–4.8 ppm range of the ^{19}F spectra of the B forms. Analysis of RNase T$_1$ fingerprints located the modified fluorodihydrouridine derivative in the A form of (FUra)tRNA$_f^{Met}$ at position 20[2] (Hills et al., 1983). To locate the position of fluorodihydrouridine in the A forms of (FUra)tRNA$_1^{Val}$ and (FUra)tRNA$_m^{Met}$, 5′-^{32}P end-labeled tRNAs were treated with aniline buffered at pH 4.5 (Peattie, 1979). These conditions induce strand scission in the tRNA at the position of the ring-opened fluorodihydrouridine residue; cleavage may also occur at m^{7}G residues (Wintermeyer & Zachau, 1970). The products of the reaction were analyzed by polyacrylamide gel electrophoresis, and the sites of cleavage were identified by comparison with a RNase T$_1$ ladder (Figure 4). In interpreting the results, note that oligonucleotides produced by RNase T$_1$ digestion terminate in a 3′-phosphate, whereas those generated by aniline cleavage terminate in a 3′-substituted ribose (Maxam, 1983). As a result, on gel electrophoresis, the aniline cleavage fragments are retarded approximately one nucleotide interval relative to the corresponding RNase T$_1$ fragments.

[2] The numbering system used is that of Sprinzl et al. (1985).

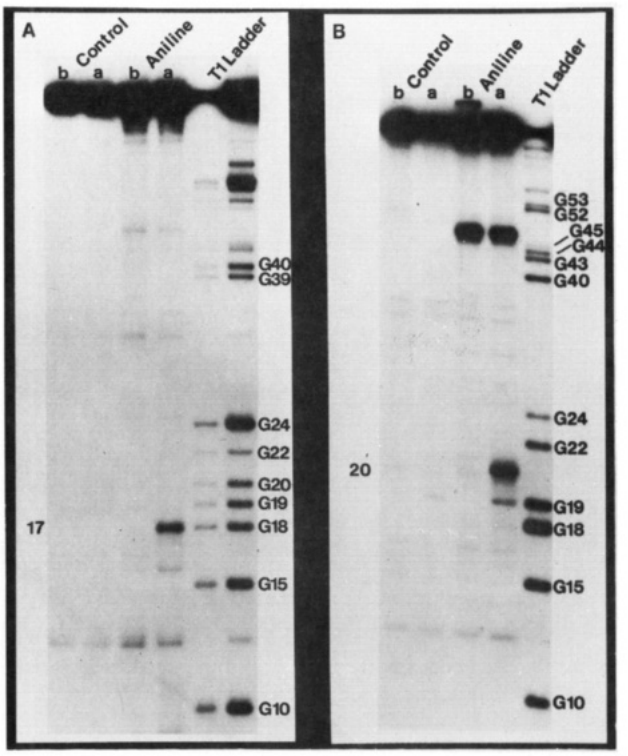

FIGURE 4: Location of 5-fluorodihydrouridine in FUra-substituted tRNAs. Autoradiograms of the gel electrophoretic analysis of fragments produced by aniline/pH 4.5 cleavage of the A (lane a) and B (lane b) forms of 5′-^{32}P end-labeled (FUra)tRNAs. (A) tRNA$_1^{Val}$; (B) tRNA$_m^{Met}$. Control samples were incubated in 50 mM sodium acetate, pH 4.5. The ribonuclease T$_1$ sequencing ladder was prepared as described under Methods.

Isoacceptor A of each tRNA species has one major aniline cleavage site not observed in isoacceptor B (Figure 4), thus locating the position of the fluorodihydrouridine residue in the tRNA. In (FUra)tRNA$_1^{Val}$, it is at position 17; in (FUra)-tRNA$_m^{Met}$, it is at position 20. Both isoaccepting forms of tRNA$_m^{Met}$ are also cleaved at the position of m^{7}G46 (Figure 4). Aniline cleaves the A form of (FUra)tRNA$_f^{Met}$ (results not shown) at position 20, confirming our previous conclusion on the location of fluorodihydrouridine in this tRNA (Hills et al., 1983). On the basis of these experiments, FUra17 in tRNA$_1^{Val}$ can be assigned to peak D (4.5 ppm) in the ^{19}F NMR spectrum of that tRNA (B form) and FUra20 to peak F (4.6 ppm) in the spectrum of (FUra)tRNA$_m^{Met}$.

The modified fluorodihydrouridine in the A form of each tRNA is located in the D loop at a position occupied by a dihydrouridine residue in the unsubstituted tRNA. It is interesting to note that tRNA$_m^{Met}$ normally contains three to four dihydrouracils in the D loop (Cory & Marcker, 1970). All of these are replaced by fluorouracil, but only the FUra residue at position 20 is reduced to fluorodihydrouracil.

^{19}F Homonuclear Overhauser Effect Studies. Assuming that (FUra)tRNA$_1^{Val}$ has a structure similar to the crystal structure of yeast tRNA^{Phe}, distance measurements,[3] made from crystallographic coordinates, show that the only fluorine atoms in the tRNA within the requisite 4–5 Å to give an appreciable ^{19}F homonuclear NOE are in FUra residues 54 and 55 (see Discussion). Observation of reciprocal NOEs between two peaks should thus aid in assigning FUra54 and -55. To minimize spin diffusion effects (Kalk & Berendsen, 1976), these studies were carried out with (FUra)tRNA$_1^{Val}$ in

[3] Distance measurements were made with the program DISPLA written by P. Briley, Department of Biochemistry, The University of Iowa, Iowa City, IA.

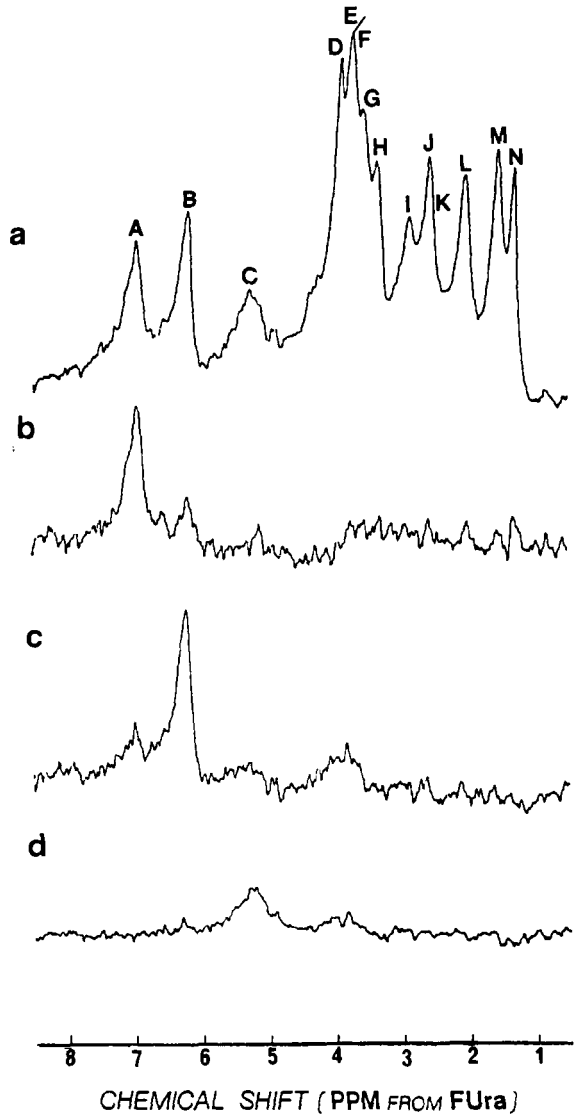

FIGURE 5: ^{19}F homonuclear Overhauser effects in the NMR spectrum of 6-deuterio-5-fluorouracil-substituted *E. coli* $tRNA_1^{Val}$ in standard buffer (see Figure 1). (a) ^{19}F NMR spectrum of 6-deuterio-FUra-substituted $tRNA_1^{Val}$ preirradiated off-resonance. (b–d) NOE difference spectra obtained on selective preirradiation of (b) peak A, (c) peak B, and (d) peak C.

which the 6-H atoms of all FUra residues are replaced by deuterium (see Methods). There are small differences between the ^{19}F NMR spectrum of the deuteriated tRNA (Figure 5a) and that of the nondeuteriated molecule (Figure 1); each resonance in the spectrum of the former is shifted upfield 0.29–0.38 ppm.

Figure 5 shows the effects of preirradiating the three well-resolved downfield peaks, A, B, and C, for 300 ms. The difference spectrum after irradiation of peak A reveals a distinct NOE to peak B (Figure 5b); a reciprocal NOE from peak B to A is also seen (Figure 5c); no NOE is observed on irradiation of peak C (Figure 5d). Because peaks A and B are close to each other in the spectrum, we tested the possibility that the results are due to power spillover by irradiating at a frequency as far downfield from peak A as peak B is upfield (219 Hz); no change in the intensity of peak A was noted under these conditions (data not shown). On the basis of these observations, we assign peaks A and B to FUra54 and -55 (see Discussion).

Thermal Denaturation Studies. In assigning peaks A and B in the ^{19}F NMR spectrum of (FUra)$tRNA_1^{Val}$ to FUra54 and -55, it is not possible to determine on the basis of ^{19}F NOE

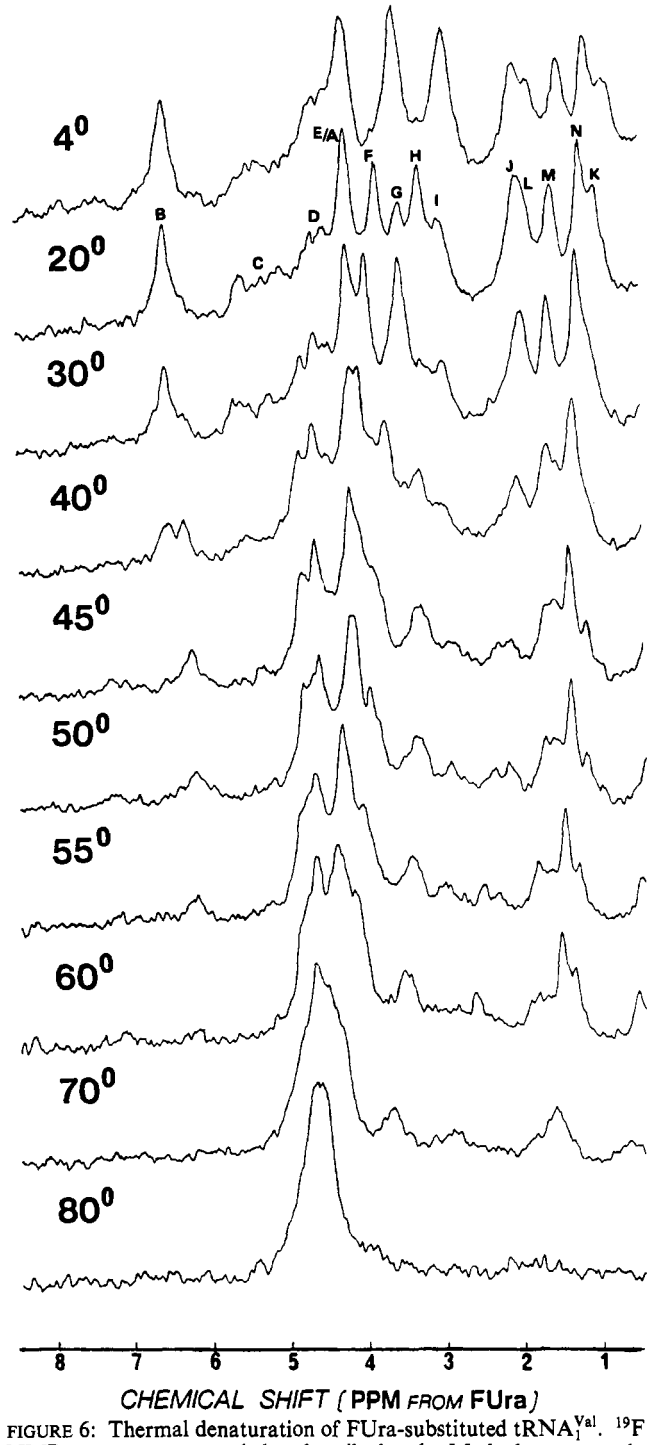

FIGURE 6: Thermal denaturation of FUra-substituted $tRNA_1^{Val}$. ^{19}F NMR spectra were recorded as described under Methods on a sample dissolved in 50 mM sodium cacodylate buffer, pH 6, and 100 mM NaCl (magnesium-free standard buffer).

experiments whether A corresponds to FUra54 and B to FUra55 or vice versa. Evidence from thermal denaturation studies, however, permits specific assignments to be made. The temperature dependence of the ^{19}F chemical shifts in the NMR spectrum of (FUra)$tRNA_1^{Val}$ is shown in Figure 6. These experiments were carried out at 100 mM NaCl in the absence of added Mg^{2+}; the spectra have been normalized to keep the intensity of the major peak at 4.5–4.8 ppm constant.

Although several interesting temperature-dependent spectral changes are noted, most relevant to the assignment problem is the observation that at temperatures between 30 and 50 °C peak B splits. With rising temperature, an increasing fraction of peak B intensity shifts from the component at 6.6 ppm to

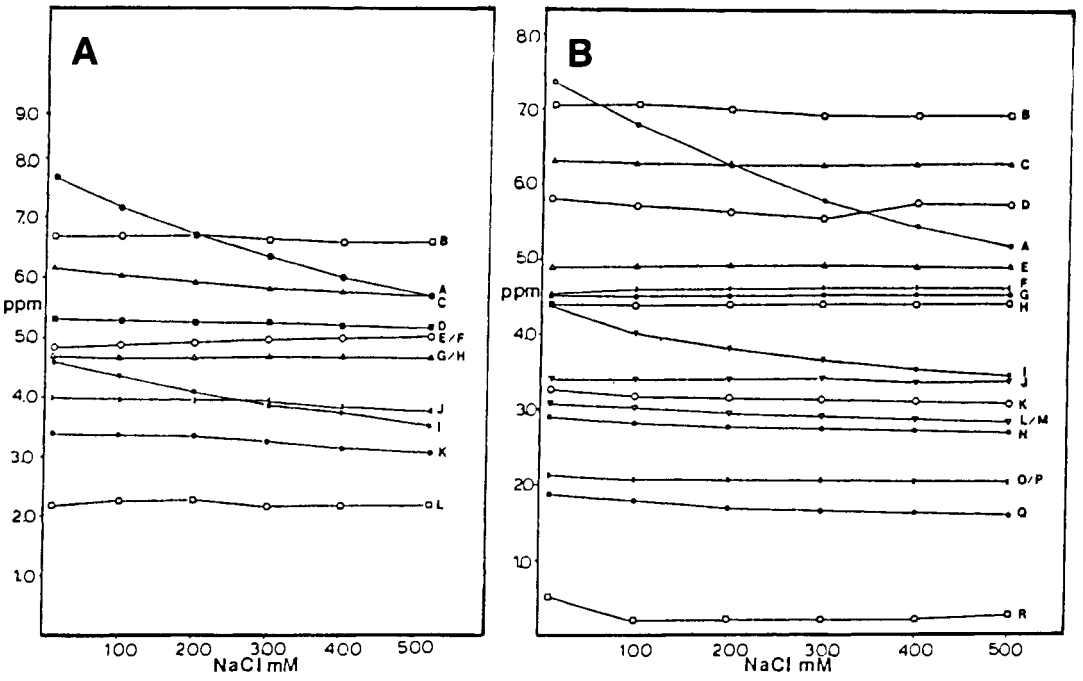

FIGURE 7: Effects of NaCl concentration on the ^{19}F NMR spectra of FUra-substituted tRNAs. (A) $tRNA_f^{Met}$ (0.40 mM); (B) $tRNA_m^{Met}$ (0.34 mM). Transfer RNAs were dissolved in standard buffer containing the indicated NaCl concentrations. ^{19}F resonances are identified as in Figure 2 and 3.

that at 6.4 ppm until, at 50 °C, more than 90% of the intensity is found at 6.4 ppm (Figure 6). Above 45–50 °C, peak intensity in this chemical shift region is lost as the corresponding portion of the tRNA structure denatures. Peak B does not split when denaturation studies are carried out in 10 mM Mg^{2+} (unpublished experiments). Because the splitting of peak B resembles the temperature-dependent splitting of T54 methyl proton and ^{13}C signals in a number of native tRNAs (Kastrup & Schmidt, 1975, 1978; Davanloo et al., 1979; Kopper et al., 1983), we assign peak B to FUra54 and peak A to FUra55 (see Discussion for additional comments).

Other spectral changes observed with increasing temperature include the downfield shift of peaks F and H between 20 and 40 °C. These ^{19}F resonances have been assigned to FUra33 and FUra34, respectively, in the anticodon loop of (FUra)-$tRNA_1^{Val}$ (Gollnick et al., 1986, 1987), and the shifts are indicative of a low-temperature change in anticodon conformation. Peaks J and L in the upfield cluster of peaks are more temperature sensitive than others in this chemical shift region. Between 25 and 30 °C, peak J shifts downfield to overlap peak L; both broaden and disappear between 40 and 55 °C.

The temperature–absorbance profile of (FUra)$tRNA_1^{Val}$, determined in the same low magnesium buffer used for the ^{19}F NMR studies, indicates that the secondary and tertiary structures of the tRNA are disrupted between 30 and 80 °C (results not shown). A low-temperature transition is observed with a T_m of 36 °C that coincides with the temperature at which the transfer of peak B intensity between 6.6 and 6.4 ppm is half-complete (see Figure 6). The UV melting curve obtained at 15 mM Mg^{2+} does not show this low-temperature transition.

Effects of Monovalent and Divalent Cations on the ^{19}F Spectra of Fluorinated tRNAs. The conformation of tRNA is known to be sensitive to the concentration of cations such as magnesium and sodium, which serve to stabilize the folded three-dimensional structure of the negatively charged polynucleotide [for reviews, see Schimmel and Redfield (1980) and also Hyde and Reid (1985)]. Changing the concentration of cations affects the ^{19}F NMR spectra of FUra-substituted tRNA. Characterization of these effects should aid in making peak assignments and could yield valuable information regarding the conformational states of tRNA in solution.

The effect of sodium ion concentration, at 15 mM Mg^{2+}, on the ^{19}F NMR spectra of FUra-substituted $tRNA_f^{Met}$ and $tRNA_m^{Met}$ is shown in Figure 7; data for (FUra)$tRNA_1^{Val}$ were reported in Hardin et al. (1986). In each case, the furthest downfield peak at low ionic strength (peak A) shifts more than 2 ppm upfield as the NaCl concentration is raised from 0 to 500 mM. A second resonance, in the central portion of each spectrum, also shifts upfield approximately 0.8 ppm with increasing NaCl concentration. These peaks are labeled F, I, and I in the spectra of FUra-substituted $tRNA_1^{Val}$ (Hardin et al., 1986), $tRNA_f^{Met}$, and $tRNA_m^{Met}$ (Figure 7), respectively. Peak F in the ^{19}F spectrum of (FUra)$tRNA_1^{Val}$ has been assigned to FUra33 in the anticodon loop (Gollnick et al., 1987), and peak I in the spectrum of fluorinated $tRNA_m^{Met}$ has been assigned to FUra36 at the 3′-position of the anticodon (Gollnick et al., 1986). The shift of these resonances with NaCl concentration thus indicates a salt-induced change in the anticodon loop. By analogy, these results suggest that peak I in the spectrum of (FUra)$tRNA_f^{Met}$ corresponds to a FUra residue in the anticodon loop, either FUra36 in the anticodon or FUra33 5′-adjacent to the anticodon.

Removal of Mg^{2+} results in major changes in the ^{19}F NMR spectrum of fluorinated tRNAs. This is shown for $tRNA_f^{Met}$ and $tRNA_m^{Met}$ in Figure 8; the results for (FUra)$tRNA_1^{Val}$ were described in Hardin et al. (1986). By following the changes in peak position as Mg^{2+} is added and assuming that the shift trends are continuous, it is possible to identify corresponding resonances in the ^{19}F spectra obtained in the presence and absence of Mg^{2+}. The largest chemical shift change observed as the ratio of Mg^{2+} to tRNA increases is the 2–3 ppm downfield shift of the resonance labeled A in the ^{19}F spectra of all three tRNAs. This is the same peak that shifts upfield with increasing Na^+ concentration (compare Figures 7 and 8). The effects of Mg^{2+} depletion are fully reversible; at Mg^{2+} levels above 50 per tRNA, the ^{19}F NMR spectra are the same as those observed at 15 mM Mg^{2+} (results not shown).

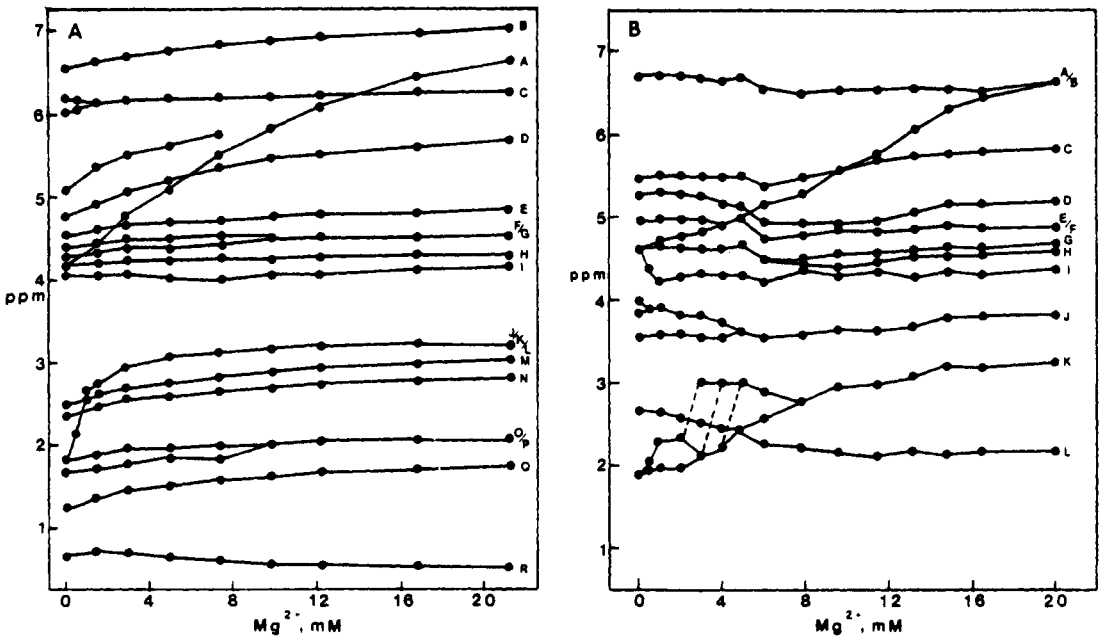

FIGURE 8: Magnesium ion concentration dependence of the chemical shifts of resonances in the ^{19}F NMR spectra of FUra-substituted tRNAs. (A) $tRNA_m^{Met}$ (0.52 mM); (B) $tRNA_f^{Met}$ (0.21 mM). Transfer RNAs were dissolved in standard buffer lacking Mg^{2+}, and various amounts of $MgCl_2$ were then added. [(FUra)$tRNA_f^{Met}$ was in magnesium-free standard buffer containing 10 mM EDTA.] Individual ^{19}F resonances are identified as in Figures 2 and 3.

DISCUSSION

The usefulness of ^{19}F NMR spectroscopy of fluorine-labeled tRNAs as a probe of tRNA solution structure depends on reliable resonance assignments to individual 5-fluorouracils in the tRNA. Incorporated FUra residues can be grouped into three classes: those participating in secondary H bonding, those involved in tertiary H bonding, and those involved in neither. Initial approaches to the assignment problem focused on assigning the ^{19}F peaks to fluorouracils in these three structural environments (Horowitz et al., 1977; Hardin et al., 1986). Because thermal denaturation of unfractionated FUra-substituted tRNA and purified (FUra)$tRNA_1^{Val}$ results in coalescence of the ^{19}F spectrum into a single peak at ca. 4.7 ppm (Figure 6) and poly(FU) (random coil) shows a single ^{19}F resonance at the same chemical shift (Hardin et al., 1986), we have assigned ^{19}F signals in the central region of the spectrum (4–5 ppm) to FUra residues in relatively unstructured (loop) regions of the tRNA; i.e., those not involved in H bonding. A number of additional experimental findings support this general assignment (Hardin et al., 1986). For example, the pH dependence of the ^{19}F NMR spectrum of (FUra)$tRNA_1^{Val}$ shows that the peaks in the central region are accessible to pH titration. These same FUra residues are highly exposed to solvent, as determined by the solvent isotope shift (SIS) seen on transferring the tRNA from H_2O to D_2O; they also readily form adducts with bisulfite, a reagent that reacts preferentially with pyrimidines in single-stranded regions of the tRNA.

Ring-current shielding effects on FUra residues in helical structures and the marked reduction of upfield peak intensities in the ^{19}F spectrum of (FUra)$tRNA_1^{Val}$ by the cyclophotoaddition of a psoralen derivative (which reacts most readily with pyrimidines in double-stranded regions) (Hardin et al., 1986) suggest assignment of upfield resonances to FUra residues in helical domains. We had earlier proposed, on the basis of the relative integrals of the downfield resonances in the ^{19}F spectra of both unfractionated FUra-substituted tRNA and (FUra)$tRNA_1^{Val}$ (Horowitz et al., 1977), that the three furthest downfield resonances correspond to FUras8, -54, and -55, which replace the invariant bases U or s^4U8, T54, and Ψ that form tertiary hydrogen bonds in all tRNAs. Support for this hypothesis is provided by the observation that the downfield resonances in the ^{19}F NMR spectrum of (FUra)-$tRNA_1^{Val}$ are the first to disappear as the temperature is raised (Figure 6). In some (Crothers & Cole, 1978) but not all tRNAs (Johnston & Redfield, 1981b; Roy & Redfield, 1983), the tertiary structure is the most thermally labile.

Results presented in this paper permit us to make several specific assignments. The firmest are based on determining, by aniline cleavage of the tRNA (Figure 4), the location of the fluorodihydrouridine derivative present in the A isoacceptor of each tRNA in place of a FUra residue in the corresponding B isoacceptor (Hills et al., 1983; Horowitz et al., 1983). This enabled us to assign peak D at 4.5 ppm in the ^{19}F spectrum of (FUra)$tRNA_1^{Val}$ to FUra17 and peak F (4.6 ppm) in the spectrum of (FUra)$tRNA_m^{Met}$ to FUra20. The peak at 4.8 ppm (peak E) in the spectrum of (FUra)$tRNA_f^{Met}$ (B form) had previously been assigned to FUra20 in that tRNA (Hills et al., 1983).

The reciprocal ^{19}F homonuclear NOE observed between resonances A and B in the ^{19}F NMR spectrum of (FUra)-$tRNA_1^{Val}$ (Figure 5) indicates that these peaks correspond to FUra residues at either position 54 or position 55. Distance estimates based on the crystal structure of yeast $tRNA^{Phe}$ show that only the fluorine atoms on FUra54 and -55 are close enough to give an appreciable NOE. The other pairs of FUra residues adjacent to each other in the nucleotide sequence are those at positions 33 and 34 and at positions 7 and 8. A sharp U turn in the anticodon loop separates the fluorines at residues 33 and 34 by more than 7 Å. FUra residues 7 and 8 are not stacked in the tRNA structure, and a large distance separates these fluorines as well. The fluorines at positions 7 and 67, on adjacent base pairs of the amino acid stem but on opposite strands, project into the major groove and are too far apart to allow an appreciable NOE. On the basis of these arguments, we can assign FUra54 and -55, which replace the invariant T54 and Ψ55, respectively, to peaks A and B (or vice versa) in the ^{19}F spectrum of (FUra)$tRNA_1^{Val}$.

^{19}F NMR melting studies provide evidence that peak B corresponds to FUra54. Thermal denaturation of (FUra)-

$tRNA_1^{Val}$, in the absence of Mg^{2+}, results in a splitting of peak B in the ^{19}F NMR spectrum, with intensity gradually shifting from 6.6 to 6.4 ppm (Figure 6). This transition, which is half-complete at ca. 36 °C, demonstrates a temperature-dependent conformational exchange between two magnetically distinct environments that is slow on the NMR time scale. The temperature-dependent splitting of peak B resembles the splitting of the T54 methyl resonances observed in both ^1H NMR (Kastrup & Schmidt, 1975, 1978; Davanloo et al., 1979) and ^{13}C NMR (Kopper et al., 1983) spectra of $tRNA_1^{Val}$ and several other tRNAs. This splitting was interpreted as a transition from the conformation seen in the crystal structure of yeast $tRNA^{Phe}$, in which the methyl group is stacked over G53 near the center of maximum shielding, to a conformation in which T54 is no longer stacked on G53 and the A58–T54 tertiary base pair is lost (Kastrup & Schimdt, 1978).

The transfer of peak B intensity from 6.6 to 6.4 ppm in the ^{19}F NMR thermal denaturation profile of FUra-substituted $tRNA_1^{Val}$, which is half-complete as ca. 36 °C (Figure 6), is paralleled by a low-temperature hyperchromicity (T_m = 36 °C) in the UV absorbance–temperature profile of $tRNA_1^{Val}$. On the basis of our assignment of peak B to FUra54, we can conclude that the T_m at 36 °C is due to a structural transition in the T loop to a less stacked conformation.

If peak B is assigned to FUra54, peak A can be assigned to FUra55 on the basis of the $^{19}F\{^{19}F\}$ NOE to peak B. Support for this assignment comes from comparison of mono- and divalent cation effects on the ^{19}F spectra of the three fluorinated transfer RNAs [Figures 7 and 8 and Hardin et al. (1986)]. In each spectrum, the furthest downfield resonance (peak A) is especially sensitive to changes in sodium (Figure 7) and magnesium ion (Figure 8) concentrations. This may be characteristic of all FUra-substituted tRNAs, suggesting that peak A corresponds to one of the invariant bases involved in maintaining the tertiary structure of the tRNA. Assignment of peak A in the ^{19}F spectrum of (FUra)$tRNA_1^{Val}$ to the FUra residue that replaces the conserved Ψ55 is consistent with this hypothesis. The results also indicate that peak A in the ^{19}F NMR spectra of (FUra)$tRNA_f^{Met}$ and (FUra)-$tRNA_m^{Met}$ may be assigned to FUra55 in these tRNAs. Redfield and collaborators (Johnston & Redfield, 1981a; Tropp & Redfield, 1981) noted a resonance in the 10.6–10.9 ppm region of the ^1H NMR spectra of yeast $tRNA^{Phe}$ and *E. coli* valine and initiator methionine tRNAs that exhibited a hypersensitivity to changes in Mg^{2+} concentration. These peaks were assigned to the N(1)-H of Ψ55 on the basis of an observed NOE from the methyl group of T54. The fluorine atom in FUra55 is in the same position as the N(1)-H of Ψ55.

In addition to the assignments described in this paper, several other specific assignments have been made on the basis of oligonucleotide binding studies. Peaks F and H at 4.5 and 3.9 ppm in the ^{19}F NMR spectrum of (FUra)$tRNA_1^{Val}$ have been assigned to FUra33 and -34 (in the anticodon loop), respectively (Gollnick et al., 1986, 1987). We have also made a preliminary assignment of peak I in the spectrum of (FUra)$tRNA_m^{Met}$ to FUra36 at the 3′-position of the anticodon in this tRNA (Gollnick et al., 1986). These assignments are in agreement with the previously discussed relationship between the chemical shift position of a resonance in the ^{19}F NMR spectrum and the structural environment of the corresponding FUra residue in the tRNA molecule.

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Transcriptional and Posttranscriptional Regulation of Tyrosine Aminotransferase by Insulin in Rat Hepatoma Cells†

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ABSTRACT: The molecular mechanisms of induction of tyrosine aminotransferase (TAT) by insulin were studied in the well-differentiated rat hepatoma cell line Fao. Incubation of Fao cells with insulin resulted in a 2-fold increase in TAT activity and TAT mRNA measured by Northern blot analysis with an oligonucleotide probe to the 5′ end of the gene. The effect of insulin on TAT activity had a lag period of 30–60 min and was maximal within 4–5 h. The insulin effect on TAT mRNA was rapid, half-maximal after 15 min, and complete within 1–2 h. Insulin dose–response curves for stimulation of TAT activity and TAT mRNA were almost identical. TAT mRNA levels and enzyme activity were also stimulated by anti-insulin receptor antibodies and dexamethasone but not by wheat germ agglutinin, concanavalin A, or phytohemagglutin. The effect of insulin on the TAT gene was further investigated by measuring the relative rate of transcription in isolated nuclei using genomic TAT clones. Insulin produced a 1.5–1.7-fold increase in the production of TAT RNA transcripts. Dexamethasone induced both TAT activity and TAT mRNA to a comparable extent. In the presence of dexamethasone, insulin produced an additional 2-fold stimulation of TAT activity but had no additional effect on the abundance of TAT mRNA. These data provide direct evidence that insulin can increase TAT activity by at least two distinct mechanisms: insulin alone appears to increase TAT activity and TAT mRNA due to a stimulation of the TAT gene transcription rate; while in the presence of glucocorticoids, insulin increases TAT activity but not TAT mRNA, suggesting an insulin effect at the posttranscriptional level.

Tyrosine aminotransferase (TAT,[1] L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) is a liver-specific gluconeogenic enzyme. The activity of tyrosine aminotransferase is regulated by various hormones including insulin, glucagon, and glucocorticoids [for a review, see Granner and Hargrove (1983)]. The mechanisms of insulin action in the regulation of TAT

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; 1× SSC, 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0; TAT, L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5); DEX, dexamethasone; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; WGA, wheat germ agglutinin; kb, kilobase(s).

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