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Isolation and Characterization of Two 5-Fluorouracil-Substituted *Escherichia coli* Initiator Methionine Transfer Ribonucleic Acids†

Diane C. Hills,‡ Matthew L. Cotten, and Jack Horowitz*

ABSTRACT: *Escherichia coli* initiator methionine tRNA labeled in vivo with 5-fluorouracil (FUra) has been isolated and characterized. The tRNA, with essentially all its uracil and uracil-derived minor bases replaced by FUra, was purified by sequential chromatography, first on diethylaminoethylcellulose (DEAE-cellulose), at pH 8.9, followed by chromatography on Sepharose 4B, using a reverse salt gradient, then on DEAE-Sephadex A-50, and finally on benzoylated DEAE-cellulose. The last step resolved two FUra-substituted $tRNA_f^{Met}$-isoaccepting species, each with a specific activity over 1500 pmol/A_{260}. Kinetic analysis shows both are aminoacylated at the same rate; apparent K_ms for the two are 0.92 and 0.94 μM, compared with 1.7 μM for normal $tRNA_f^{Met}$. Chromatographic differences between the two forms of fluorinated $tRNA_f^{Met}$ persist after aminoacylation, and the two tRNAs are not interconverted by denaturation and renaturation. The isoacceptors have nearly identical nucleoside composition, and both contain 7-methylguanosine and 2′-*O*-methylcytidine as the only modified nucleosides. Analysis of complete RNase T_1 digests of the two methionine tRNAs shows that they differ in only one oligonucleotide. The sequence ^{20}FpApGp, derived from the dihydrouridine loop and stem region, which is found in one of the isoaccepting forms of the tRNA, is replaced by an oligonucleotide containing adenine and guanine, but no FUra in the other. A modified FUra, with the properties of a 5-fluoro-5,6-dihydrouracil derivative, is detected in this tRNA. ^{19}F NMR spectra of the two species of FUra-substituted initiator tRNA show 9–10 resolved resonances for the 12 FUra residues incorporated. The spectra differ primarily in the shift of one peak in the form lacking the sequence ^{20}FpApGp, from 4.8 ppm downfield from free FUra (=0 ppm) to 14.9 ppm upfield from the standard.

Transfer RNAs isolated from 5-fluorouracil (FUra)[1]-treated *Escherichia coli* are highly substituted with the fluoropyrimidine (Horowitz & Chargaff, 1959). Incorporated FUra replaces not only uridine but also uridine-derived minor nucleosides such as pseudouridine, ribothymidine, 5,6-dihydrouridine, and 4-thiouridine (Johnson et al., 1969; Lowrie & Bergquist, 1968; Kaiser, 1972). The resulting modification-deficient tRNAs, with a few exceptions (Ramberg et al., 1978), remain functional in protein synthesis and have proved to be quite useful for examining the role(s) of modified nucleosides in tRNA structure and function (Horowitz et al., 1974; Ofengand et al., 1974; Chinalli et al., 1978). Detailed studies with purified, highly substituted *E. coli* $tRNA_1^{Val}$ have shown that incorporation of 5-fluorouridine has little if any effect on the conformation of this tRNA (Horowitz et al., 1974). Furthermore, its rate of aminoacylation is normal (Horowitz et al., 1974), and it is active in all steps of protein synthesis in vitro (Ofengand et al., 1974).

In addition to examining the biochemical function of fluorine-substituted tRNAs, we have been exploring the use of fluorine-19 nuclear magnetic resonance as a probe of the molecular structure and dynamics of tRNA in solution. Fluorine NMR is well suited to such studies because of the high sensitivity of the ^{19}F nucleus and the large range of its chemical shifts. Since nuclear magnetic resonance permits observation of individual nuclei at different molecular sites, each FUra residue can, in principle, serve as a reporter of structural changes in its vicinity. Because FUra is distributed

† From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011. *Received August 13, 1982.* Journal Paper No. J-10720 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA; Project No. 2245. This investigation was sponsored by grants from the U.S. Public Health Service (GM 09042) and the American Cancer Society (NP-296).

‡ Present address: Department of Biochemistry, University of Osteopathic Medicine, Des Moines, IA 50302.

[1] Abbreviations: FUra, 5-fluorouracil; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)-tetraacetic acid; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; PEI–cellulose, poly(ethylenimine)–cellulose; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

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throughout the fluoropyrimidine-substituted tRNA, structural changes in all parts of the molecule can be monitored, including those occurring in single-stranded regions not readily accessible to study by proton NMR of hydrogen-bonded base pairs [recently reviewed by Reid (1981)]. ^{19}F NMR should be particularly useful in studies of the detailed mechanism of tRNA recognition by aminoacyl-tRNA synthetases and other proteins, since the protein does not contribute to the spectrum of the tRNA–protein complex. Our initial efforts were focused on *E. coli* $tRNA_1^{Val}$. The ^{19}F NMR spectrum of this tRNA exhibits 11–13 well-resolved peaks (Horowitz et al., 1977; Cohn et al., 1981), indicating that most of the 14 incorporated fluorines are located in distinct chemical environments. As yet, however, none of the resonances have been assigned to individual FUra residues.

To further clarify the effects of incorporated fluoropyrimidines on tRNA structure and function, and to facilitate interpretation of the ^{19}F NMR spectra, we have extended these studies to include additional fluorinated transfer RNAs. In the course of isolating the initiator methionine tRNA from FUra-treated *E. coli* (Hills, 1981), two major forms of fluorouracil-substituted $tRNA_f^{Met}$ were separated. The present study describes some structural and functional characteristics of these molecules. They were found to differ at only one FUra residue, located at position 20, in the dihydrouridine loop region of the tRNA, the position normally occupied by 5,6-dihydrouridine in *E. coli* $tRNA_f^{Met}$. The isoacceptor lacking FUra at this position contains a modified FUra with the properties of a 5-fluoro-5,6-dihydrouracil derivative. Comparison of the ^{19}F NMR spectra of the two isoaccepting species has permitted preliminary assignment of a peak 4.8 ppm downfield from FUra to the FUra residue at position 20.

Experimental Procedures

Materials

Unfractionated *E. coli* tRNA was purchased from either Schwarz/Mann or Plenum Scientific Research; purified normal *E. coli* $tRNA_f^{Met}$ came from Boehringer-Mannheim Biochemicals. 5-Fluorouracil was donated by Hoffmann-La Roche, and calcium leucovorin was a gift from Lederle Laboratories. Sephacryl S-200, DEAE-Sephadex A-50, and Sepharose 4B were products of Pharmacia. Phosphocellulose (Cellex-P) and BD-cellulose were supplied by Bio-Rad Laboratories, DEAE-cellulose (DE-32) was a Whatman product, and cellulose thin-layer sheets (5502) were from EM Laboratories. Cellulose (MN 300) was obtained from Brinkmann Instruments, and poly(ethylenimine), 50% w/w, was from Kodak Laboratory Chemicals. Poly(ethylenimine)–cellulose sheets, 0.5 mm in thickness, were prepared in the laboratory and pretreated as described by Randerath & Randerath (1966). Ribonuclease T_1, RNase T_2, and RNase A were bought from Sigma; bacterial alkaline phosphatase (BAPF) and snake venom phosphodiesterase (VPH) were from Worthington Biochemical Corp. Radioactive amino acids were supplied by New England Nuclear or ICN Pharmaceuticals, and ^3H-labeled KBH_4 was from Amersham Corp. Purified *E. coli* methionyl-tRNA synthetase was a gift from Dr. Mildred Cohn (University of Pennsylvania).

Methods

Purification of $tRNA_f^{Met}$. The growth of *E. coli* B with 5-fluorouracil was described in Horowitz et al. (1974). Bulk tRNA was prepared from FUra-treated bacteria by phenol extraction of whole cells (Zubay, 1966), and contaminating ribosomal RNA was removed by chromatography on Sephacryl S-200 (Horowitz et al., 1974). FUra-containing tRNA was separated from normal tRNA on DEAE-cellulose (2.5 × 90 cm) as described by Kaiser (1969). The column was developed at room temperature with a 8000-mL concave gradient from 0.325 to 0.6 M NaCl in 0.02 M Tris-HCl, pH 8.9. Methionine acceptor activity was located by aminoacylation assay; fractions containing highly FUra-substituted $tRNA_f^{Met}$ were pooled, and the RNA was freed of salts by precipitation with ethanol. This RNA was then dissolved in 0.01 M sodium acetate buffer, pH 4.5, containing 0.01 M $MgCl_2$ and 1.5 M $(NH_4)_2SO_4$ and loaded onto a column of Sepharose 4B (2 × 94 cm) equilibrated with the same buffer at 4 °C. Transfer RNA was eluted with 3000 mL of a reverse salt gradient of 1.5–0.0 M $(NH_4)_2SO_4$ (Holmes et al., 1975; Colantuoni et al., 1978). Pooled methionine tRNA was either dialyzed against distilled water to remove salts and concentrated by ethanol precipitation or desalted on DEAE-cellulose as described by Gillam & Tener (1980). The RNA was further fractionated at room temperature on DEAE-Sephadex A-50 (1.5 × 160 cm), using a 1800-mL linear gradient from 0.425 to 0.5 M NaCl in 0.02 M Tris-HCl buffer, pH 7.5, containing 8 mM $MgCl_2$. A final purification step involved chromatography at 4 °C on BD-cellulose (0.9 × 120 cm), using a 650-mL linear gradient of 0.4–0.07 M NaCl in 0.01 M sodium acetate, pH 4.5, and 0.01 M $MgCl_2$.

Aminoacylation and Formylation of $tRNA_f^{Met}$. The methionine acceptance of column fraction obtained during the purification procedure was measured in a reaction mixture containing 0.1 M Hepes, pH 7.5, 0.01 M KCl, 0.01 M ATP, 0.001 M dithiothreitol, 0.02 M magnesium acetate, (6–8) × 10^{-5} M [^{14}C]methionine (50 mCi/mmol) or [^3H]methionine (0.2–1.0 Ci/mmol), 300 μg/mL partially purified aminoacyl-tRNA synthetases (Muench & Berg, 1966), and varying amounts of tRNA in a final volume of 0.1 mL. Reactions were run at 37 °C for 30 min and stopped by addition of 5% cold trichloroacetic acid. After 10 min in the cold, the precipitate was collected on Millipore filters, dried, and counted in a liquid scintillation spectrometer in a toluene–PPO–POPOP mixture.

The kinetics of methionyl-tRNA formation were determined in a reaction mixture containing 0.04 M Hepes, pH 7.5, 0.15 M KCl, 0.002 M ATP, 0.001 M dithiothreitol, 0.007 M magnesium acetate, 1 × 10^{-4} M EDTA, 0.1 mg/mL bovine serum albumin, 8 × 10^{-5} M [^{14}C]methionine (51.4 mCi/mmol), 180 pmol of purified methionyl-tRNA synthetase (Fayat et al., 1977), kindly supplied by Dr. Mildred Cohn, and varying amounts of tRNA. Reaction mixtures were preincubated at 25 °C for 1 min before addition of the enzyme. After incubation for the appropriate times, the reaction was stopped and prepared for counting as described earlier.

Assays for the formylation of methionyl-tRNA were carried out by charging the tRNA in the presence of 6 × 10^{-4} M 10-formyltetrahydrofolate. Sufficient transformylase was present in partially purified synthetase preparations to catalyze the reaction. The extent of formylation was determined by incubation at 37 °C with 0.01 M $CuSO_4$–0.2 M sodium acetate, pH 5.5, as described by Schofield & Zamecnik (1968). Incubations were carried out for 20 min, which gave good discrimination between N-blocked and unblocked methionyl-tRNA. 10-Formyltetrahydrofolate was prepared from calcium leucovorin (5-formyltetrahydrofolate) by the method of Dubnoff & Maitra (1971).

Polyacrylamide–Urea Gel Electrophoresis. Polyacrylamide gels (10–20%) [acrylamide:bis(acrylamide) ratio of 20:1], prepared in a 0.02 M Tris–acetate buffer, pH 8.0, containing 0.001 M EDTA and 7 M urea, were cast in a vertical slab gel apparatus (15 × 12 × 0.15 cm). Transfer RNA samples,

mixed with a dye solution (40% sucrose, 7 M urea, and 0.025% bromophenol blue), were loaded on the gels, and electrophoresis was carried out for 4 h at 12 mA. The gels were then stained overnight in 1% pyronin Y dissolved in 15% acetic acid and destained in 7.5% acetic acid.

Determination of Nucleoside Composition. The nucleoside composition of purified tRNAs was determined by the tritium derivative method of Randerath, E., et al. (1972) and Randerath, K., et al. (1974) as described earlier (Horowitz et al., 1974). Several FUra-substituted $tRNA_f^{Met}$ samples were also analyzed by HPLC, using the method of Davis et al. (1979). These analyses were kindly carried out by Drs. Gherke and Kuo (University of Missouri).

Ribonuclease T_1 Oligonucleotide Maps. Complete RNase T_1 digests of tRNA were prepared as described by Randerath et al. (1980), by incubating $3A_{260}$ units of the RNA in a reaction mixture (60 μL) containing 3600 enzyme units of RNase T_1 in 20 mM Tris-HCl, pH 7.6, for 2.5 h at 38 °C. The resulting oligonucleotides were separated by two-dimensional chromatography on prewashed PEI–cellulose TLC plates. These were developed with stepwise salt gradients as described by Chen & Roe (1978), except that the concentrations of LiCl (in 0.3 M Tris-HCl, pH 7.9, and 7.5 M urea) used in the first dimension were 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1.0 M. The concentrations of lithium formate (pH 3.5 in 7.5 M urea) used in the second dimension were 0.1, 0.8, 1.2, 1.6, and 2.2 M. The TLC plates were cut after chromatography in the first dimension to permit longer development of the portion of the plate containing the more slowly migrating oligonucleotides, thus increasing their resolution. Individual spots were visualized under a UV lamp, scraped from the TLC plates, and loaded onto small columns of phosphocellulose built in Pasteur pipets. Oligonucleotides were eluted with 4.0 M pyridinium formate, pH 4.0, lyophilized to dryness, and stored at −20 °C. The nucleoside composition of each oligonucleotide was determined by the scaled-down version 2 of the tritium derivative procedure detailed in Randerath et al. (1980). A mixture of unlabeled nucleoside trialcohol markers was added to the sample before TLC, and the location of individual constituents on the chromatograms was detected under a UV light.

Colorimetric Determination of Dihydropyrimidines. The dihydropyrimidine content of tRNA was determined by the colorimetric microassay of Jacobson & Hedgcoth (1970). This method is based on the alkaline conversion of dihydropyrimidines to open-ring ureidopropionic acid derivatives which are quantified by a colorimetric assay specific for the ureido group (Hunninghake & Grisolia, 1966). Ten microliters of 1 N KOH was added to a 0.1-mL sample. After incubation at 37 °C for 30 min, 0.05 mL of concentrated H_2SO_4 and 0.1 mL of a mixture containing *N*-phenyl-*p*-phenylenediamine diacetyl monoxime (Hunninghake & Grisolia, 1966) were added. The assay tubes were heated at 95 °C for 5 min and then transferred to a 50 °C water bath for an additional 5 min before the addition of 0.1 mL of 10^{-3} M $FeCl_3$ in concentrated H_2SO_4. After the tubes were cooled to room temperature, the A_{550} was determined. Standard curves were prepared with 5,6-dihydrouridine and 5′-deoxy-5-fluoro-5,6-dihydrouridine. The amount of tRNA assayed was calculated from the A_{260}, assuming a value of $A^{0.1\%}_{260nm} = 24$ and a molecular weight of 26 000.

^{19}F NMR Spectroscopy. Transfer RNA samples (0.5–3.5 mg) were dissolved in 0.3 mL of buffer containing 50 mM sodium cacodylate, pH 6.0, 10 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, and 10% D_2O as a lock signal and transferred to a Wilmad 529A-10 NMR microtube. This was suspended in a 10-mm NMR sample tube and surrounded with sample buffer containing 1 mM FUra as an external standard. ^{19}F NMR spectra were obtained on a Bruker W300 pulsed FT NMR spectrometer operating at 282 MHz. Chemical shifts are reported relative to FUra, and shifts to higher shielding are indicated by negative numbers. After each set of experiments, the activity of the tRNA sample was checked by aminoacylation assay and possible degradation monitored by polyacrylamide gel electrophoresis.

Results

Purification of FUra-Substituted $tRNA_f^{Met}$. Incorporation of 5-fluorouracil modified the properties of $tRNA_f^{Met}$ sufficiently so that published methods for the isolation of *E. coli* initiator tRNA (Dube & Marcker, 1969) no longer sufficed, and a four-step procedure to purify FUra-substituted $tRNA_f^{Met}$ was developed. In the first step, unfractionated tRNA was chromatographed on DEAE-cellulose at pH 8.9 (Kaiser, 1969; Horowitz et al., 1974). Normal tRNAs elute early and are separated from FUra-substituted molecules, which emerge as a broad second peak (Figure 1A). Four methionine-accepting tRNAs were detected; the first two (fractions 150–230) were eluted with the unsubstituted tRNAs and contained normal $tRNA_f^{Met}$ and $tRNA_m^{Met}$. These were clearly separated from the FUra-substituted methionine tRNAs, which eluted as two well-resolved peaks, with some indication that each was heterogeneous (Figure 1A). The major FUra-containing methionine tRNA (Figure 1A, fractions 300–370) was more than 90% formylatable, as determined by the method of Schofield & Zamecnik (1968); the second fluorinated methionine-accepting tRNA (Figure 1A, fractions 410–465) could not be formylated ($tRNA_f^{Met}$).

Fractions containing FUra-substituted $tRNA_f^{Met}$ were pooled, and the tRNA, which at this stage had a specific activity of 155 pmol/A_{260}, was further purified on Sepharose 4B, using a reverse concentration gradient of $(NH_4)_2SO_4$ (Holmes et al., 1975; Colantuoni et al., 1978). One major peak of methionine-accepting activity was found (Figure 1B); a minor species of methionine tRNA, eluting later than the major component, was also observed. On rechromatography, this minor species again separated from the major form of (FUra)$tRNA_f^{Met}$, but no attempt was made to purify this component further. The enriched $tRNA_f^{Met}$, specific activity 660 pmol/A_{260}, was then chromatographed on DEAE-Sephadex A-50 (Figure 1C), and the single peak of methionine tRNA (specific activity 1300 pmol/A_{260}) was further purified on BD-cellulose. Two isoaccepting species of methionine tRNA were resolved at this step (Figure 2), the first, isoacceptor A, having a specific activity of 1600 pmol/A_{260} and the second, isoacceptor B, 1550 pmol/A_{260}. Both isoacceptors were formylated to the extent of 108% (form A) and 93% (form B). Approximately equal amounts of the two species of (FUra)$tRNA_f^{Met}$ were present in the experiment shown. However, the relative amounts varied from one experiment to another, and subsequent preparations contained more of the B component.

Recent results have indicated that each isoacceptor can be further resolved into two components by chromatography on RPC-3 (Weeren et al., 1972). These have the nucleoside composition expected of FUra-substituted $tRNA_{f1}^{Met}$ and $tRNA_{f3}^{Met}$ (Egan et al., 1973) with the latter containing no m^7G (M. L. Cotten and J. Horowitz, unpublished experiments). Chromatography of (FUra)$tRNA_{f1}^{Met}$ on RPC-5 failed to give additional purification. However, a partial resolution of isoacceptor A into two peaks was noted; isoacceptor B chroma-

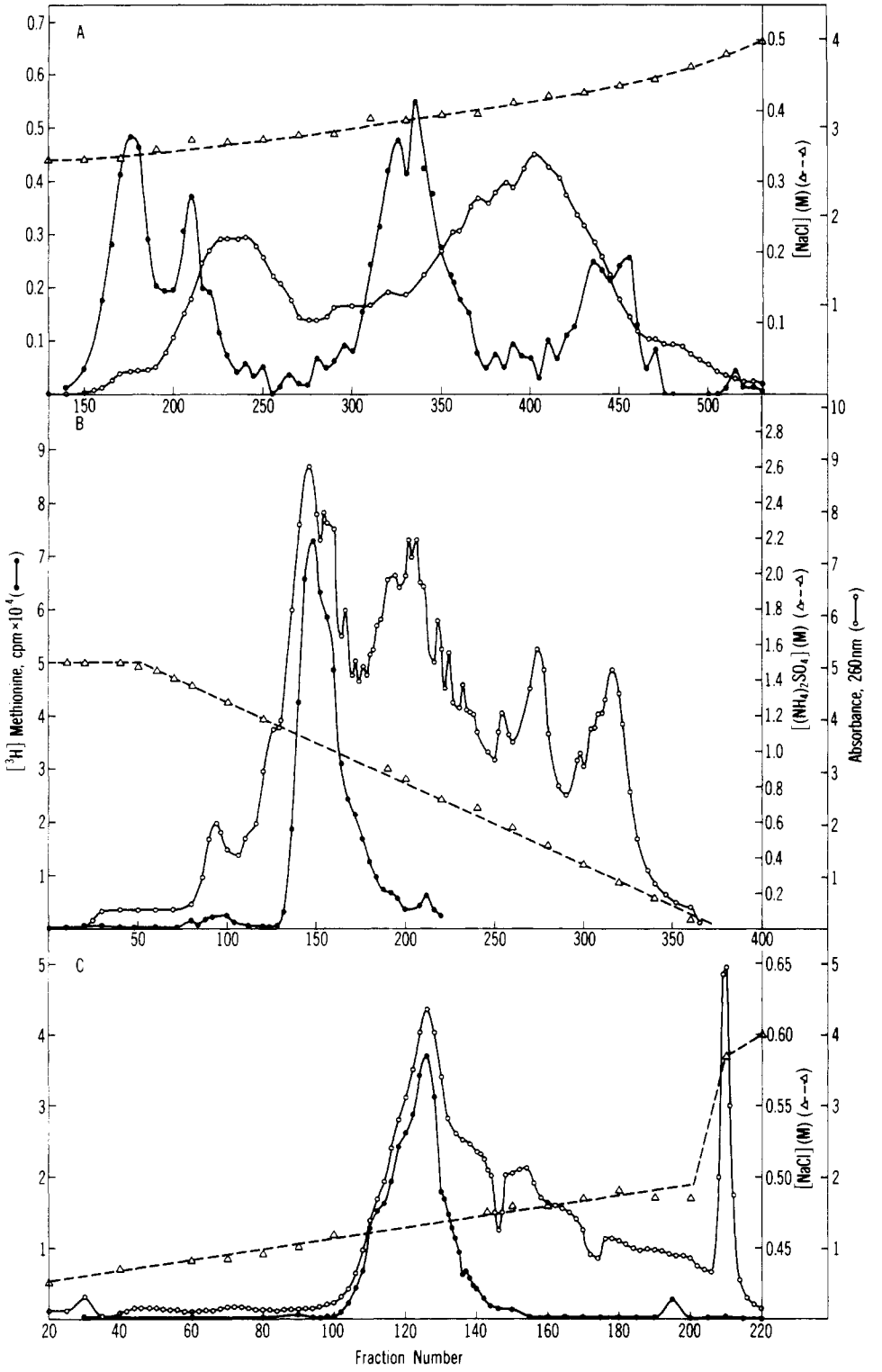

FIGURE 1: Chromatographic purification of FUra-substituted $tRNA_f^{Met}$. (A) 10250 A_{260} units of unfractionated *E. coli* FUra-containing tRNA were chromatographed on a column of DEAE-cellulose as described under Experimental Procedures. Fractions of 25 mL were collected at a flow rate of 120 mL/h. (B) The FUra-substituted methionine tRNA-rich pool from DEAE-cellulose (9400 A_{260} units) was charged onto Sepharose 4B and the column developed with a decreasing ammonium sulfate gradient as described under Methods. Fractions of 15 mL were collected at a flow rate of ca. 40 mL/h. (C) 1600 A_{260} units of partially purified $tRNA_f^{Met}$ were chromatographed on DEAE-Sephadex A-50 as described in the text. Fractions of 8 mL were collected at a flow rate of 8 mL/h. (○) Absorbance at 260 nm; (●) methionine acceptor activity; (Δ) salt concentration.

tographed as a single peak on RPC-5.

Kinetic Parameters of the Aminoacylation of FUra-Substituted $tRNA_f^{Met}$. Both FUra-containing initiator tRNAs were clearly recognizable by their cognate synthetase and by methionyl-tRNA transformylase since they were purified on the basis of their ability to be aminoacylated, and the resulting methionyl-$tRNA_f^{Met}$s were fully formylatable. To study the interaction of synthetase and tRNA in more detail and to determine whether the two isoaccepting species of (FUra)-$tRNA_f^{Met}$ differ in their ability to accept methionine, we compared the kinetics of aminoacylation of normal and FUra-substituted $tRNA_f^{Met}$. Purified methionyl-tRNA

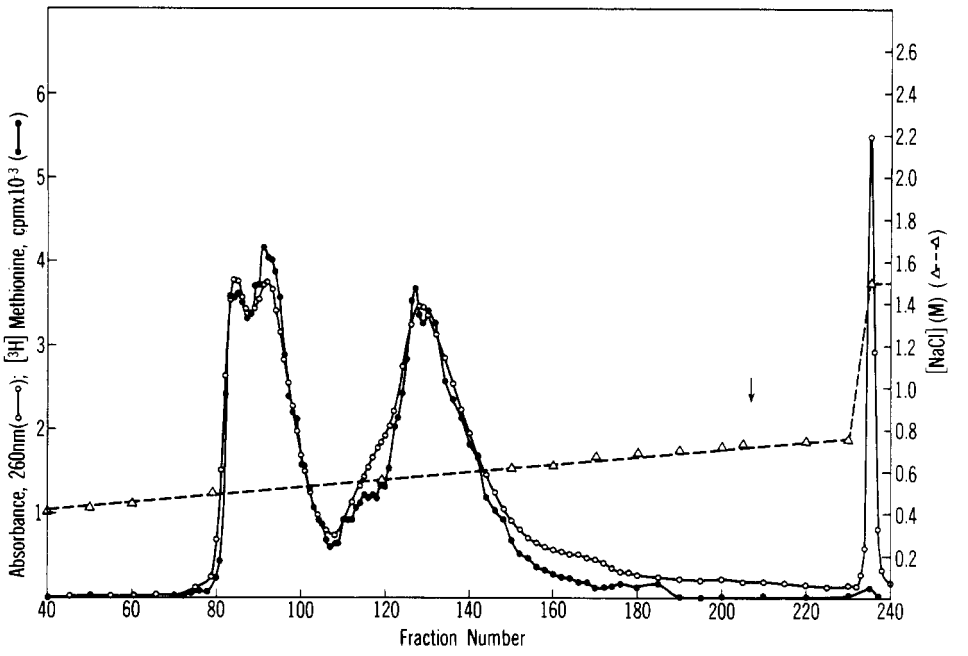

FIGURE 2: Separation of two forms of FUra-substituted $tRNA_f^{Met}$ on BD-cellulose. 600 A_{260} units of FUra-substituted $tRNA_f^{Met}$ recovered from DEAE-Sephadex A-50 (Figure 1C) were applied to a BD-cellulose column and chromatographed as described under Methods. The flow rate was 15 mL/h. Each fraction contained 3 mL and was assayed for the absorbance at 260 nm (○) and methionine acceptor activity (●). (△) NaCl concentration.

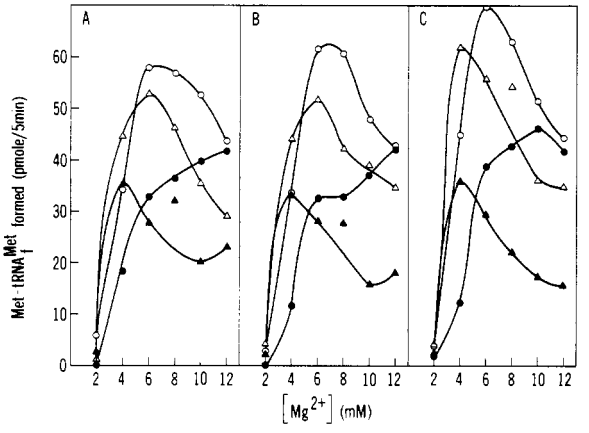

FIGURE 3: Effect of magnesium and KCl concentrations on the initial rate of aminoacylation of $tRNA_f^{Met}$. (A) (FUra)$tRNA_f^{Met}$ form A; (B) (FUra)$tRNA_f^{Met}$ form B; (C) normal $tRNA_f^{Met}$. Reactions were carried out as described under Methods in 10 (▲), 75 (△), 150 (○), and 300 mM (●) KCl. Transfer RNA concentration was 4 μM, and the concentration of purified methionyl-tRNA synthetase was 372 pM.

synthetase (Fayat et al., 1977), kindly supplied by Dr. Mildred Cohn, was used in these experiments.

Optimal conditions for the rate studies with each of the three tRNAs, normal methionine tRNA and the two forms of FUra-substituted $tRNA_f^{Met}$, were determined by examining the magnesium ion requirement of the reaction at various KCl concentrations. The results, presented in Figure 3, were obtained by using a constant amount of tRNA (4 μM) and the same limiting concentration of purified enzyme (372 pM). It is clear that the magnesium optima at different KCl concentrations are the same for each of the three tRNAs. Highest rates were obtained at 0.15 M KCl, and the magnesium optimum at this salt concentration was 6–8 mM. These results are similar to those reported by Blanquet et al. (1973) for normal $tRNA_f^{Met}$.

Apparent K_m and V values for each of the three tRNAs were measured at 25 °C in 150 mM KCl and 7 mM magnesium acetate. Results (Table I) were calculated by a weighted linear regression analysis (Wilkinson, 1961; Barnes & Waring, 1980) of the data points in double-reciprocal plots. The apparent K_m found for normal $tRNA_f^{Met}$ (1.7 μM) differed somewhat from the value of 3.7 μM reported by Blanquet et al. (1973), determined under similar conditions. Apparent K_ms for the two FUra-substituted tRNAs were identical and slightly lower than that for normal initiator tRNA (Table I).

Table I: Kinetic Parameters for Aminoacylation of Normal and FUra-Substituted $tRNA_f^{Met}$

tRNA species	K_m (μM)	V^a (s^{-1})
normal $tRNA_f^{Met}$	1.7 ± 0.3	6.8 ± 0.6
(FUra)$tRNA_f^{Met}$ (A)	0.92 ± 0.08	5.8 ± 0.2
(FUra)$tRNA_f^{Met}$ (B)	0.94 ± 0.13	5.7 ± 0.3

[a] Values expressed as the number of moles of aminoacyl-tRNA formed per mole of enzyme per second at 25 °C. Enzyme concentration was 180 pM.

Electrophoretic and Chromatographic Properties of FUra-Substituted $tRNA_f^{Met}$. To better characterize the FUra-substituted $tRNA_f^{Met}$s and to determine the basis for chromatographic differences between the two isoaccepting forms, we examined the electrophoretic and chromatographic behavior of free and aminoacylated tRNAs more fully. Each tRNA gave a single band on electrophoresis in 15% polyacrylamide gels under denaturing conditions (7 M urea). Both forms of FUra-substituted methionine tRNA migrated more slowly than normal $tRNA_f^{Met}$, and two bands were observed in mixtures of normal and analogue-substituted tRNA (data not shown). Mixtures of the two forms of FUra-substituted $tRNA_f^{Met}$ could not be clearly resolved on single-dimensional gels, or by two-dimensional electrophoresis on 10 or 20% gels in the pH 8.3 borate buffer system described by Ikemura & Dahlberg (1973). Heating at 65 °C for 5 min in 6 M urea or treatment for 1 h with 1 M glyoxal in 50% dimethyl sulfoxide to irreversibly denature the RNA (McMaster & Carmichael, 1977) revealed no hidden breaks in either form

Table II: Nucleoside Composition of Normal and FUra-Substituted $tRNA_f^{Met}$ [a]

nucleoside	normal tRNA		(FUra)tRNA (A)		(FUra)tRNA (B)		expected [b] composition
	^3H derivative	HPLC	^3H derivative	HPLC	^3H derivative	HPLC	
A	14.1	14.1	14.3	13.8	14.1	13.7	14
G	23.4	23.9	23.0	24.5	23.4	24.2	24
C	25.5	25.6	26.0	25.5	25.1	25.1	25
U	9.05	8.2	0.76	0.63	1.35	1.16	8 + 1 [c]
5FU			10.7	11.0	10.6	11.2	(12)
5FC			0.38	ND	0.41	ND	0
hU	1.03	ND	0.08	ND	0.10	ND	1
m^5U	0.96	1.02	0.02	0.01	0.05	0.03	1
Ψ	0.89	1.01	0.08	0.13	0.11	0.05	1
m^7G	1.01 [d]	0.51	0.83 [d]	0.59	0.81 [d]	0.45	1
s^4U	c	0.80	c	0.29	c	0.32	1
Cm	ND	0.91	ND	0.58	ND	0.75	1

[a] Nucleoside composition was determined by both the ^3H-derivatization procedure of Randerath, E., et al. (1972) and Randerath, K., et al. (1974) and by HPLC (Davis et al., 1979). Values are expressed as residues per mole. [b] Dube & Marcker (1969). [c] 4-Thiouridine is recovered as uridine in the ^3H-labeling procedure. [d] Corrected for 64% recovery.

of the tRNA. Subsequent electrophoresis on denaturing gels showed only single bands with the mobility of intact tRNA.

Chromatographic differences between forms A and B persisted even after aminoacylation. (FUra)$tRNA_f^{Met}$ (A), aminoacylated with ^{14}C-labeled methionine, is eluted from BD-cellulose earlier than the B form, charged with ^3H-labeled methionine (results not shown). This effectively rules out the loss of the 3′-terminal adenosine or other modification at the 3′ end of the tRNA as the basis for the difference between forms A and B. Furthermore, the two forms of FUra-substituted $tRNA_f^{Met}$ do not represent interconvertible conformers. Denaturation of either isoacceptor by heating at 50 °C in the presence of EDTA, followed by renaturation in magnesium-containing buffer, does not convert form A to B or vice versa.

Nucleoside Composition. The nucleoside composition of normal and analogue-substituted methionine tRNAs was determined by two different methods, the ^3H-substitution procedure of Randerath, E., et al. (1972) and Randerath, K., et al. (1974) and HPLC (Davis et al., 1979). These gave very similar results (Table II). As previously described for valine tRNA (Horowitz et al., 1974), almost all the uracil-derived nucleosides were substituted by FUra; 90% or more of the uridine, dihydrouridine, pseudouridine, and ribothymidine was replaced. Only small differences in nucleoside composition between the two isoaccepting forms of FUra-substituted $tRNA_f^{Met}$ were noted. Form B had a somewhat higher content of uridine than form A, and the degree of substitution of the minor uridine-derived bases was also marginally lower in form B (Table II). The degree of replacement of 4-thiouridine (s^4U), determined by HPLC, was only ca. 60%, but this is probably the result of difficulties in determining this constituent by HPLC because of the low response factor of s^4U. The other nucleoside constituents were present in the same proportions in all three tRNAs. Cm was found in both forms of FUra-substituted $tRNA_f^{Met}$; the B component had a somewhat higher content of this nucleoside than form A. Both isoacceptors contained m^7G.

Analysis of Two-Dimensional RNase T_1 Fingerprints. For the detection of possible sequence differences between the two isoaccepting species of FUra-substituted $tRNA_f^{Met}$, the products of complete RNase T_1 digestion were separated by two-dimensional chromatography on PEI–cellulose. The $tRNA_{f1}^{Met}$ fraction isolated by RPC-3 chromatography was used in these experiments to simplify interpretation of results. Each oligonucleotide was analyzed by the ^3H-labeling method (Randerath et al., 1980) and its sequence deduced from the nucleoside composition by reference to the known sequence of

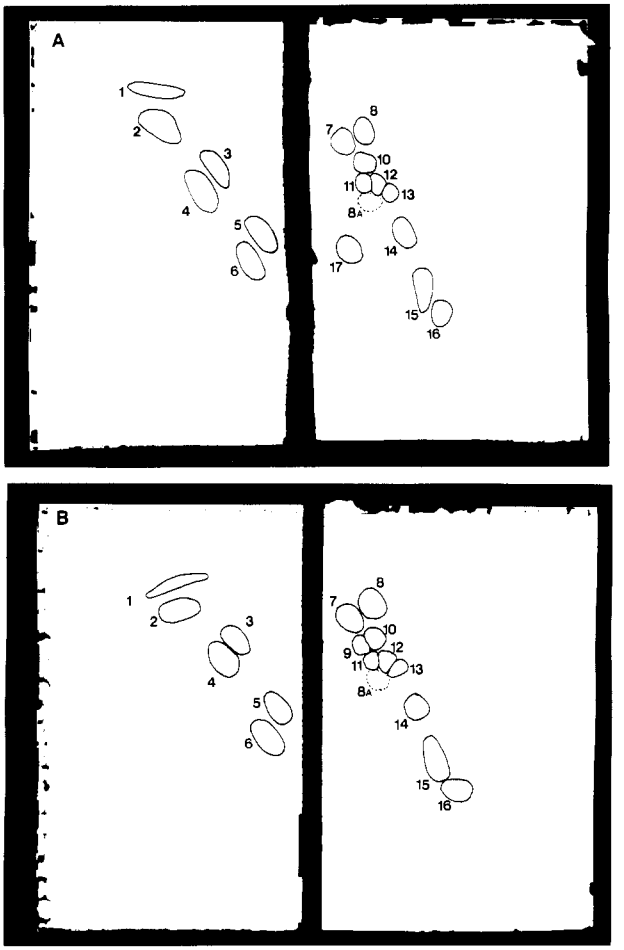

FIGURE 4: Oligonucleotide map of RNase T_1 digests of FUra-substituted $tRNA_f^{Met}$. Oligonucleotides in a complete RNase T_1 digest of tRNA were chromatographed on PEI–cellulose as described under Methods. First dimension (LiCl gradient), from right to left; second dimension (lithium formate gradient), from bottom to top. The TLC plate was cut after the first dimension, and the two halves were chromatographed separately in the second dimension to increase the resolution of the more slowly migrating oligonucleotides. (A) (FUra)$tRNA_f^{Met}$ form A; (B) (FUra)$tRNA_f^{Met}$ form B.

E. coli $tRNA_f^{Met}$ (Dube & Marcker, 1969). Analysis of the fingerprint of the B form of fluorinated methionine tRNA (Figure 4 and Table III) showed it to contain all the expected RNase T_1 oligonucleotides with 5-fluorouridine replacing uridine and uridine-derived minor nucleosides. Only the expected RNase T_1 hydrolysis products were observed, indicating

Table III: Analysis of Oligonucleotides Produced by Complete Ribonuclease T_1 Digestion of FUra-Substituted $tRNA_f^{Met}$

oligonucleotide no.	nucleoside content[a] form A	nucleoside content[a] form B	oligonucleotide sequence[b]	molar ratio[c] form A	molar ratio[c] form B	molar ratio[c] expected
T1	C(1.1), A(1)	C(0.9), A(1)	C-A-A-C-C-A_{OH}	1.0	0.8	1
T2	G(1)	G(1)	Gp	8.5	10.3	9
T3	C(1.0), G(1)	C(1.1), G(1)	C-Gp	1.3	1.5	1
T4	A(1.0), G(1)	A(1.0), G(1)	A-Gp	1.0	1.0	1
T5	A(1.1), C(1.2), G(1)	A(1.2), C(1.2), G(1)	C-A-Gp	1.1	1.2	1
T6	A(2.0), G(1)	A(2.1), G(1)	A-A-Gp	1.0	0.9	1
T7	F(1.0), G(1)	F(1.0), G(1)	F-Gp	0.9	1.0	1
T8	m^7G(0.6), C(1.1), F(1.0), G(1)	m^7G(0.7), C(1.1), F(1.0), G(1)	m^7G-F-C-Gp	0.8	0.5	1
T8a	C(1.5), F(0.7), G(1)	C(1.5), F(0.8), G(1)		0.5	0.7	
T9	not detected	A(0.8), F(1.0), G(1)	F-A-Gp		1.0	1
T10	C(1.1), F(1.0), G(1)	C(1.0), F(0.9), G(1)	F-C-Gp	2.3	1.8	2
T11	C(4.6), G(1)	C(4.1), G(1)	C-C-C-C-C-Gp	0.8	0.6	1
T12	C(2.6), F(0.8), G(1)	C(2.4), F(0.8), G(1)	C-F-C-Gp or	1.1	1.3	1
T13	C(2.0), F(1.0), G(1)	C(2.2), F(1.0), G(1)	C-C-F-Gp	1.1	0.8	1
T14	C(1.2), G(1)	C(1.2), G(1)	pC-Gp	1.2	0.9	1
T15	A(3.3), C(4.3), F(2.2), G(1)[e]	A(3.5), C(4.7), F(2.0), G(1)[e]	(Cm)-F-C-A-F-A-A-C-C-C-Gp	0.8	0.5	1
T16	A(3.0), C(3.1), F(2.8), G(1)	A(3.3), C(3.4), F(2.9), G(1)	F-F-C-A-A-A-F-C-C-Gp	0.6	0.7	1
T17	A(0.9), G(1)	not detected		0.5		

[a] Determined by the tritium derivative procedure (Randerath, E., et al., 1972; Randerath, K., et al., 1974). Results are expressed relative to the 3′-terminal nucleoside and represent the average of two independent determinations. [b] The sequence of each fragment was deduced from the composition by reference to the known primary structure of $tRNA_f^{Met}$ (Dube & Marcker, 1969). No attempt was made to differentiate the sequence isomers C-F-C-Gp and C-C-F-Gp (fragments T12 and T13). [c] Molar ratios were calculated relative to the average of the most frequent count rates of the 3′-terminal trialcohols. [d] Oligonucleotide 8a is probably a degradation product of 8 from which m^7G is missing. The sum of 8 + 8A gave a molar ratio of ca. 1.0 in all samples analyzed. [e] Cm is not detected by the tritium derivative procedure.

that this tRNA has the same sequence as normal $tRNA_{f1}^{Met}$ except for the 5-fluorouridine substitutions. The oligonucleotide map of isoacceptor A differs from that of B in only one oligonucleotide; fragment T9 is missing, and a new fragment, T17, is present (compare panels A and B of Figure 4). T9 has the sequence FpApGp (Table III). Oligonucleotide T17, like T9, contains equimolar amounts of adenosine and guanosine, but no detectable levels of 5-fluorouridine were found (Table III). Evidence indicates that T17 is larger than a dinucleotide. Removal of the 3′-terminal phosphate with bacterial alkaline phosphatase followed by ^{3}H labeling of the 3′ end by the usual procedures results in a product that migrates more slowly than a dinucleotide derivative on PEI–cellulose in the Tris-HCl (pH 8.0)–8.5 M urea solvent system described by Randerath et al. (1980), which resolves oligonucleotides according to chain length. We were unable to identify the additional nucleotide by the ^{3}H labeling and mapping procedures used here (but seen the next section). There was no indication of a 2′-*O*-methyl-substituted nucleotide in T17; the oligonucleotide was completely hydrolyzed by RNase T_2.

The sequence FpApGp (oligonucleotide T9), found in isoacceptor B but replaced by oligonucleotide T17 in A, occurs only once in (FUra)$tRNA_f^{Met}$ (Table III and Figure 5), in the dihydrouridine loop and stem of the molecule; its FUra residue is situated at position 20[2] in the tRNA. The two isoaccepting forms of FUra-substituted $tRNA_f^{Met}$ thus differ only in the region of the dihydrouridine arm, with isoacceptor B having a FUra residue at position 20 that is absent in isoacceptor A. Preliminary results of direct sequence analysis of the two fluorinated initiator tRNAs, using the controlled single hit hydrolysis procedure of Stanley & Vassilenko (1978) as modified by Gupta & Randerath (1979), confirm that their sequences are identical with that of normal $tRNA_f^{Met}$ (FUra replacing Ura and uracil-derived bases) except for an unidentified nucleotide in the dihydrouridine loop of the A form of FUra-substituted $tRNA_f^{Met}$ (B. A. Roe, D. P. Ma, and J. Horowitz, unpublished experiments).

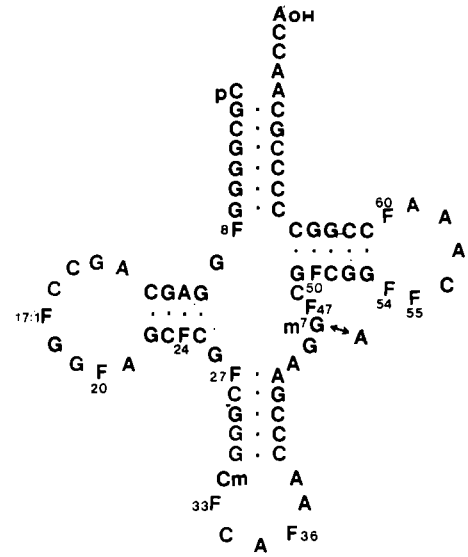

FIGURE 5: Nucleotide sequence (cloverleaf structure) of *E. coli* $tRNA_f^{Met}$, with uracil and uracil-derived nucleosides replaced by 5-fluorouracil (F).

Evidence for a Fluorodihydropyrimidine Derivative in Isoacceptor A of FUra-Substituted $tRNA_f^{Met}$. *E. coli* initiator methionine tRNA normally has 5,6-dihydrouridine at position 20 (Dube & Marcker, 1969). This, together with the observation of an upfield resonance characteristic of 5,6-saturated fluoropyrimidines in the ^{19}F NMR spectrum of the A form of (FUra)$tRNA_f^{Met}$ (see next section), suggested that the unidentified nucleotide in isoacceptor A might be a fluorodihydropyrimidine derivative. To test this possibility, we determined the dihydropyrimidine content of normal and FUra-substituted $tRNA_f^{Met}$ by the colorimetric microassay of

[2] The numbering system used is that of Gauss et al. (1979).

Table IV: Dihydropyrimidine Content of Normal and 5-Fluorouracil-Substituted $tRNA_f^{Met}$

tRNA species	dihydropyrimidine content: colorimetric method[a]	dihydropyrimidine content: ^3H-derivative method[b]
normal $tRNA_f^{Met}$	1.05 ± 0.04	1.03 ± 0.06
(FUra)$tRNA_f^{Met}$ (A)	1.05 ± 0.2	0.03 ± 0.01
(FUra)$tRNA_f^{Met}$ (B)	0.19 ± 0.08	0.05 ± 0.005

[a] Dihydropyrimidine content determined by the colorimetric method of Hunninghake & Grisolia (1966), as modified by Jacobson & Hedgcoth (1970). Values are expressed as residues per mole. [b] Dihydrouridine determined by the ^3H-derivatization procedure of Randerath, E., et al. (1972) and Randerath, K., et al. (1974). Values are expressed as residues per mole.

Jacobson & Hedgcoth (1970). The results are shown in Table IV. Normal $tRNA_f^{Met}$ has, as expected, one residue of dihydropyrimidine per mole. Only low levels of dihydropyrimidine were detected in the B form of FUra-substituted $tRNA_f^{Met}$. However, isoacceptor A was found to contain 1 mol of dihydropyrimidine per mol of tRNA (Table IV). Of the constituents found in RNA, only dihydropyrimidines yield color in the assay (Jacobson & Hedgcoth, 1970). Fluorodihydropyrimidines also react; a model compound, 5′-deoxy-5-fluoro-5,6-dihydrouridine, gives essentially the same color yield as dihydrouridine (P. Gollnick and J. Horowitz, unpublished experiments).

In contrast to the results of the colorimetric assay, determination of dihydrouridine in the A form of FUra-substituted $tRNA_f^{Met}$ by the ^3H-derivative method of Randerath, E., et al. (1972) and Randerath, K., et al. (1974) detected less than 0.1 residue per mol (Tables II and IV). Similar low levels of 5,6-dihydrouridine were found in isoacceptor B, while normal $tRNA_f^{Met}$ contains the expected 1 mol per mol of tRNA (Tables II and IV). The ^3H-labeling procedure involves separation of individual nucleoside trialcohols by TLC and specifically determines 5,6-dihydrouridine, while the colorimetric assay measures total dihydropyrimidine content. Isoacceptor A of (FUra)$tRNA_f^{Met}$, therefore, contains a dihydropyrimidine other than 5,6-dihydropyrimidine, most likely a fluorodihydrouridine derivative. ^{19}F NMR evidence supports this conclusion (see next section and Discussion). While insufficient amounts of oligonucleotide T17 were available for analysis, the results of the oligonucleotide mapping and gel sequencing experiments indicate that the dihydropyrimidine detected in isoacceptor A must be at position 20 of the tRNA.

Fluorine-19 NMR Spectra of FUra-Substituted $tRNA_f^{Met}$. Having determined that the difference between the two isoaccepting forms of FUra-substituted $tRNA_f^{Met}$ involves the FUra residue at position 20, we compared the ^{19}F NMR spectra of the two species of tRNA to obtain additional evidence for a fluorodihydropyrimidine derivative in isoacceptor A and to assign a ^{19}F resonance to the FUra residue at position 20 in isoacceptor B. The spectra are shown in Figure 6 and were determined on samples containing only the $tRNA_{f1}^{Met}$ component isolated by chromatography on RPC-3. At pH 6.0 in 15 mM Mg^{2+} and 10 mM NaCl, nine resolved resonances are observed in the spectrum of isoacceptor B, all downfield from the standard, FUra (Figure 6B). The spectrum of isoacceptor A differs from that of B at three points (compare spectra A and B of Figure 6). Most striking is the appearance of a peak ca. 15 ppm upfield from FUra. The position of this resonance does not shift on hydrolysis of the tRNA with either RNase T_2 or alkali (results not shown), suggesting that the

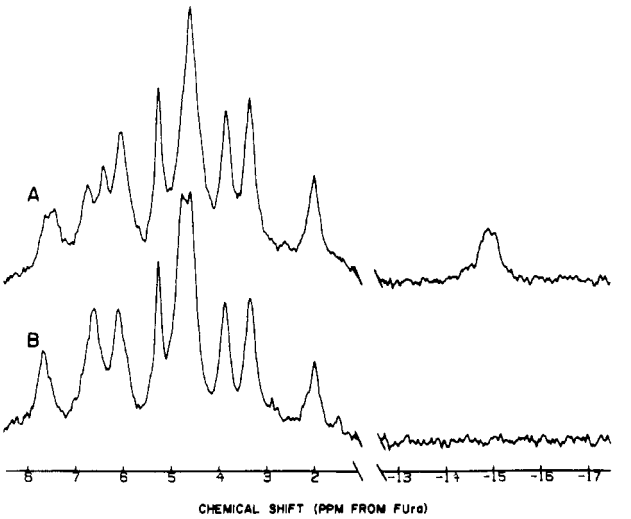

FIGURE 6: ^{19}F NMR spectra of FUra-substituted $tRNA_f^{Met}$. The spectra were recorded at 282 MHz in the FT mode as described under Methods. (A) (FUra)$tRNA_f^{Met}$ form A; (B) (FUra)$tRNA_f^{Met}$ form B. The samples were dissolved in 50 mM sodium cacodylate, pH 6.0, containing 10 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, and 10% D_2O. Chemical shifts are given in parts per million from 5-fluorouracil.

upfield shift is not due to the structure of the intact tRNA molecule. A large upfield shift is expected on saturation of the 5,6-double bond of FUra (see Discussion), and our observation of a resonance at −15 ppm is consistent with the finding of a fluorodihydrouridine derivative in isoacceptor A (Table IV). A second difference in the ^{19}F NMR spectra of the two fluorinated $tRNA_f^{Met}$'s is the presence in the spectrum of the B form of a resonance 4.8 ppm downfield from FUra not seen in the spectrum of the A form (Figure 6). Differences are also observed in the region of the spectrum 6–7 ppm downfield from FUra. Isoacceptor A has two peaks in this part of the spectrum, one at 6.4 ppm and the other at 6.8 ppm, while isoacceptor B has one resonance at 6.6 ppm that falls midway between the two; some spectra of isoacceptor A exhibit a third peak in this region at 6.6 ppm.

^{19}F NMR spectra were obtained at pH 6 in order to minimize effects due to the dissociation of the N(3)-H of incorporated FUra. 5-Fluorouridine has a pK_a of 7.57 (Wempen et al., 1961), and FUra incorporated into poly(FU) has a pK_a of 8.1–8.3 (Szer & Shugar, 1963; Massoulié et al., 1966). A chemical shift 1.6 ppm to lower shielding occurs on dissociation of the proton from the fluorinated nucleoside (C. C. Hardin and J. Horowitz, unpublished experiments). Increasing the pH from 6 to 7 causes as much as a 0.3-ppm downfield shift of some ^{19}F resonances, while others do not shift (results not shown). Ionic strength also has a differential effect on the position of peaks in the spectrum. Raising the NaCl concentration from 10 to 100 mM results in a 0.5-ppm shift to higher shielding of the peak farthest downfield in the spectrum; considerably smaller shifts in other resonances are also observed (C. C. Hardin and J. Horowitz, unpublished experiments).

Discussion

E. coli $tRNA_f^{Met}$ is the second FUra-substituted transfer RNA to be purified. Like the first, $tRNA_1^{Val}$ (Horowitz et al., 1974), the highly substituted molecule, with 90% or more of its uracil and uracil-derived minor constituents replaced by FUra, remains fully recognizable by its cognate synthetase as well as by methionyl-tRNA transformylase (Figure 3 and Table I). Fluorinated $tRNA_f^{Met}$ occurs in at least two forms, both fully active in accepting methionine. The ability of these

tRNAs to function in the initiation of protein synthesis is not yet known and is currently under investigation. It is interesting to note that other FUra-substituted tRNAs can also be separated into two forms in much the same way as fluorinated $tRNA_f^{Met}$. Purified (FUra)$tRNA_m^{Met}$ was resolved into two components on BD-cellulose, and, recently, two species of fluorinated $tRNA_1^{Val}$ were separated by chromatography on RPC-5 (C. C. Hardin and J. Horowitz, unpublished experiments).

In our attempts to determine the molecular basis for the difference between the two isoaccepting forms of FUra-substituted methionine tRNA, we were able to rule out loss of all or part of the 3′-CCA terminus because the tRNAs were still separable on BD-cellulose after aminoacylation. Furthermore, the two forms are not conformational isomers, since it was not possible to interconvert them by heat denaturation and renaturation. Few differences in the nucleoside composition of the two isoaccepting methionine tRNAs are evident (Table II). Both contain m^7G; thus, neither represents the minor species, $tRNA_{f3}^{Met}$, in which m^7G at position 46 is replaced by A (Egan et al., 1973). In fact, *both* of the FUra-substituted $tRNA_f^{Met}$ isoacceptors isolated from BD-cellulose are mixtures of $tRNA_{f1}^{Met}$ and $tRNA_{f3}^{Met}$. Analysis of the RNase T_1 digestion products derived from the FUra-substituted methionine tRNAs (Table III) makes clear that both isoacceptors have the expected 5′- and 3′-terminal sequences. Processing of the FUra-substituted tRNA precursors thus seems to be normal and unaffected by the presence of FUra or the absence of many modified nucleosides. Furthermore, the electrophoretic differences observed between analogue-substituted and normal $tRNA_f^{Met}$ are not due to additional residues present at either terminus of the molecule.

Sequence determination of the two forms of fluorinated $tRNA_f^{Met}$ by analysis of RNase T_1 fingerprints (Table III) and by direct gel sequencing demonstrates that they differ only at position 20, in the dihydrouridine loop of the tRNA. Isoacceptor B contains FUra at this position, while several lines of evidence indicate that the base at position 20 in the A form is a fluorodihydrouracil derivative. The strongest evidence for this is the detection, by the colorimetric assay of Jacobson & Hedgcoth (1970), of 1 mol of dihydropyrimidine per mol of tRNA in isoacceptor A but not in B (Table IV). This assay determines the ureido group produced by the alkali-catalyzed ring opening of dihydropyrimidines; only the dihydropyrimidine constituents of tRNA give a position reaction (Jacobson & Hedgcoth, 1970). Since direct analysis for 5,6-dihydrouridine by the ^3H-derivative method shows that both isoaccepting forms of FUra-substituted $tRNA_f^{Met}$ contain only small amounts of this constituent (Tables II and IV), it is most probable that the positive result obtained with the colorimetric procedure is due to a fluorodihydrouridine derivative in isoacceptor A. Observation of a ^{19}F resonance upfield from FUra in the ^{19}F NMR spectrum of the A form lends support to this conclusion.

Saturation of the 5,6-double bond of FUra or its derivatives leads to large upfield ^{19}F chemical shifts. This has been demonstrated for the bisulfite adduct (Sander & Deyrup, 1972) and for 5-fluoro-6-methoxy-5-methyl-5,6-dihydrouracil and 5-fluoro-6-methoxy-5,6-dihydrouracil (Byrd et al., 1978). The upfield ^{19}F resonance observed in the NMR spectrum of the A form of FUra-substituted $tRNA_f^{Met}$ (Figure 6) is, however, not at the position expected of fluorodihydrouridine. Fluorodihydrouridine derivatives having a proton at position 5 exhibit chemical shifts 30–40 ppm to higher shielding with respect to FUra, while the chemical shifts of derivatives with a carbon substituent at position 5 are less pronounced, 5–8 ppm upfield from FUra (Byrd et al., 1978; Lewis et al., 1980). Our own results with the model compound 5′-deoxy-5-fluoro-5,6-dihydrouridine show a peak in the ^{19}F NMR spectrum 31.5 ppm upfield from FUra (M. L. Cotten and J. Horowitz, unpublished experiments). The position of the upfield peak in fluorinated $tRNA_f^{Met}$ form A is intermediate between these two regions, and it is unlikely to be due to intact fluorodihydrouridine in the tRNA. Dihydrouridines are known to be labile to alkali, undergoing a ring-opening reaction to yield ureidopropionic acid derivatives (Cohn & Doherty, 1956; Chaudhuri et al., 1958). We have recently found, by following the loss of absorbance at 235 nm, that 5′-deoxy-5-fluoro-5,6-dihydrouridine is more unstable than 5,6-dihydrouridine and has a half-life in 0.1 N NaOH of only 15 s compared to 2–3 min for dihydrouridine. Furthermore, the fluorodihydrouridine is labile at pH 8.9 ($t_{1/2}$ = 26–30 min) while dihydrouridine itself is stable under these conditions (J. Horowitz, unpublished experiments). When the model fluorodihydrouridine is exposed to 0.05 M NaOH for 15 min or to pH 8.9 buffer for longer periods, the ^{19}F resonance at −31.5 ppm shifts to ca. −16 ppm, close to the position of the peak observed in the spectrum of isoacceptor A of fluorinated $tRNA_f^{Met}$. Our procedure for tRNA purification involves an extended period of chromatography at pH 8.9 to separate normal and FUra-substituted tRNA (see Methods). Under these conditions, fluorodihydrouridine present in the tRNA would undergo ring opening to yield a ureidopropionic acid derivative that undoubtedly accounts for the ^{19}F resonance at −15 ppm. This derivative will also give a positive reaction in the colorimetric assay for dihydropyrimidines.

Recent experiments with unfractionated FUra-substituted tRNA that had not been exposed to high pH conditions support these conclusions. The ^{19}F NMR spectrum of this tRNA has a multiplet ^{19}F resonance 32–33 ppm upfield from FUra (M. L. Cotten and J. Horowitz, unpublished experiments), in the region expected from an intact fluorodihydrouridine substituent in the tRNA. Incubation of the tRNA at pH 8.9 results in a downfield shift of this peak to −15 ppm.

The ^{19}F NMR spectra of the two FUra-substituted methionine tRNAs (Figure 6) show 9–10 well-resolved peaks derived from the 12 fluorouracils or fluorouracil derivatives in each molecule. It is thus possible to resolve almost all the incorporated FUra residues. Assigning individual resonances in the ^{19}F spectrum, however, remains a fundamental problem. Reliable assignments would permit the interactions of tRNA with proteins and other ligands to be studied by ^{19}F NMR spectroscopy. Because the two fluorinated methionine isoacceptors differ only at position 20, form B having a 5-fluorouridine at that position which is missing from A, it should be possible to assign ^{20}FUra by identifying a resonance present in the ^{19}F NMR spectrum of isoacceptor B but absent from that of A. Comparison of the two spectra shows that isoacceptor B has a resonance 4.8 ppm downfield from FUra that is missing from the spectrum of A. It is tempting to assign this resonance to ^{20}FUra. Such an assignment is consistent with earlier proposals based on studies with unfractionated FUra-substituted tRNA and with purified *E. coli* $tRNA_1^{Val}$ (Horowitz et al., 1977; Cohn et al., 1981). These suggested that the major peaks in the 5-ppm range are derived from FUra residues in non-hydrogen-bonded regions of the tRNA since heat denaturation of (FUra)tRNA (Cohn et al., 1981) and of FUra-substituted 5S RNA (Marshall & Smith, 1977) results in a collapse of the spectrum and the appearance of a single resonance at this position. The FUra at position 20

is located in a loop region of the tRNA and is not involved in secondary or tertiary interactions (Woo et al., 1980). Additional support for the assignment of the resonance at 4.8 ppm to the FUra residue in the dihydrouridine loop comes from the observation that the ^{19}F NMR spectra of the two isoaccepting species of FUra-substituted $tRNA_1^{Val}$, separated by RPC-5 chromatography, differ only in that one isoacceptor has a peak 4.5 ppm downfield from FUra that is replaced by a ^{19}F resonance 14.7 ppm upfield from the standard in the other isoacceptor (C. C. Hardin and J. Horowitz, unpublished experiments).

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Registry No. Methionyl-tRNA synthetase, 9033-22-1.

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